CONTRACEPTIVE AND ENDOMETRIAL ASPECTS
OF PROGESTINS AND ANTI-PROGESTINS

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University of Edinburgh
2003
Declaration

I declare that this thesis has been composed by myself, and the studies presented here are the results of my own independent investigations, unless otherwise acknowledged.

The content of this thesis has not and is not being currently submitted for candidature for any other degree, diploma or professional qualification.

Dharani Kosala Hapangama
BSc(Hons) MBChB
25th April 2003
Abstract

The main focus of the studies undertaken as part of this thesis was to develop new methods of contraception, while broadening our current understanding of the anti-fertility potentials of two progesterone receptor modulators: antiprogesterone compound mifepristone, and the synthetic progestin Levonorgestrel (LNG). We suggest that LNG taken immediately before ovulation acts as an emergency contraceptive (EC) by delaying or preventing ovulation. We have demonstrated that the combination of a home-use fertility monitor with once a month administration of mifepristone (especially if mifepristone is administered at the early luteal phase) is an acceptable contraceptive option with minimal side effects. Our investigations have explained some characteristics of non-compliant behaviour. We have shown that the microelectronic monitoring systems provide objective information no other monitoring technique can produce. This information offers the opportunity to make the optimum use of potentially effective treatments while validating research evidence. We have further illustrated, that this microelectronic system of Luteinizing Hormone (LH) detection can replace laboratory assays in monitoring frequently changing hormone levels in contraceptive research because it is easy to use and provides reliable data on compliance. Finally, we conclude that mifepristone induce endometrial-shedding & vaginal bleeding, in the mid-luteal phase by a mechanism involving both Prostaglandin Dehydrogenase (PGDH) and Cyclo-oxygenase – 2 (COX-2) to increase local Prostaglandin (PG) levels in the endometrium. These studies provide information which will be useful in the development of novel methods of contraception involving a “once a month” pill.
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin Biotin Peroxidase Complex</td>
</tr>
<tr>
<td>ABC-HRP</td>
<td>Avidin Biotin Peroxidase Complex – horseradish peroxidase</td>
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<tr>
<td>ALT</td>
<td>Alanine amino transferase</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>COC</td>
<td>Combined oral contraceptives</td>
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<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase - 2</td>
</tr>
<tr>
<td>CPEFM</td>
<td>ClearPlan Easy Fertility Monitor</td>
</tr>
<tr>
<td>cPR</td>
<td>Chicken PR</td>
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<tr>
<td>CVF</td>
<td>Cervico-vaginal fluid</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>E3G</td>
<td>Oestrone-3-glucuronide</td>
</tr>
<tr>
<td>EC</td>
<td>Emergency contraception</td>
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<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>FSH</td>
<td>Follicular Stimulating Hormone</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin releasing hormone</td>
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<tr>
<td>HRE</td>
<td>Hormone regulatory elements</td>
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<tr>
<td>hsp</td>
<td>Heat shock proteins</td>
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<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
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<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LNG</td>
<td>Levonorgestrel</td>
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<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
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<td>MUC-1</td>
<td>Mucin glycoprotein 1</td>
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<tr>
<td>NET</td>
<td>Norethisterone</td>
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<tr>
<td>P</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with Tween</td>
</tr>
<tr>
<td>PC</td>
<td>Pill counts</td>
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<td>PG</td>
<td>Prostaglandins</td>
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<tr>
<td>PGDH</td>
<td>Prostaglandin 15-Dehydrogenase</td>
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<td>Abbreviation</td>
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<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PRₐ</td>
<td>Progesterone receptor type A</td>
</tr>
<tr>
<td>PRₜ</td>
<td>Progesterone receptor type B</td>
</tr>
<tr>
<td>PRKO</td>
<td>Progesterone receptor knock-out</td>
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<tr>
<td>RIA</td>
<td>Radio-immuno assay</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
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<tr>
<td>SR</td>
<td>Self report</td>
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<tr>
<td>StAR</td>
<td>Sterido-genic acute regulatory protein</td>
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<tr>
<td>TAF</td>
<td>Transactivation function</td>
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<tr>
<td>UN</td>
<td>United Nations</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1

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1.1 General Introduction

The world population reached 6 billion on 12\textsuperscript{th} October 1999, according to the best guess of demographers. It has doubled in less than forty years, and if the decline in fertility rates falters, could yet double during our lifetime (United Nations (UN) population division 1999). The changes that occurred during the last century including the early menarche and age of first intercourse, late marriage, with many women choosing not to have children (mirrored by the highly significant decline in total fertility rate per woman (UN 1999a)), mean that the women of today are burdened more than ever “from the tyranny of excessive fertility” (Baird 1965). In order to avoid an unwanted pregnancy, sexually active women may have to adhere with a contraceptive regimen all their reproductive life – i.e. over a 40-year period. World Health Organization (WHO) reports a significant change in the use of contraception in the world, but we are still failing to meet the demand of millions of couples who want to plan their families and prevent pregnancies. In 1994, it was estimated that approximately 300 million couples in the world were using a method of contraception either that they are dissatisfied with or is unacceptable to them (UN population division 1994). Fuelled by women’s increased education and awareness of the need for contraception, the customer base for contraception is likely to expand significantly over the next decade (UN 1995). Since we are in the era of the largest cohort of reproductive aged population in history, consequences of even a small difference in unwanted fertility will be catastrophic. Although the steroid hormonal regimens dominated the female methods of reversible contraceptives over the last 40 years, side effects have severely affected their acceptability. This provides the incentive for the pursuit of novel alternative ‘non-hormonal’ methods of contraception.

Progesterone controls most, if not all, of the key events surrounding the complex regulation of normal female reproductive function. Thus, a substance with anti-progesterone properties should be versatile not only as a contra-gestive but also as a contraceptive. Mifepristone binds with high affinity for progesterone receptor and
antagonises the actions of progesterone at its receptor level (Baulieu 1989a). It has been successfully used to induce medical termination of pregnancy in the UK, France, Sweden and in China. Our study described herein explored the feasibility of using mifepristone as a once-a-month contraceptive pill (Chapter 3).

Widespread use of effective emergency contraceptives (EC) is estimated to prevent millions of abortions in the world (Glasier & Baird 1997 Review). The synthetic progestin Levonorgestrel (LNG) was licensed in the UK early in the millennium, as a new EC agent. It has already been used for this purpose in several other countries. Despite this, the mechanism by which LNG acts when given in this manner is unknown. In order to explain the post-coital contraceptive mechanism we investigated the effect of LNG on the menstrual cycle (Chapter 2).

Non-adherence to a contraceptive method interferes with its efficacy and disrupts the evaluation of results in a research setting (Rudd et al. 1991; Urquhart 1991). Although a problem of great importance, the realities of patient non-compliance with contraceptives are still poorly understood. The literature on non-compliance with the use of different contraceptive methods is limited. If at all, very little progress is made in either accurately detecting, or predicting non-compliance. We sought to obtain insight into the adherence behaviour of women taking part in a contraceptive trial assessing the feasibility of administering once a month mifepristone (Chapter 4).

The frequent and regular laboratory assays of steroid hormones in either blood or in urine had traditionally played a major role in determining the fertile period of the cycle. In contraceptive research these assays have been used as the standard method of timing the ovulation. This approach is not only inconvenient for the volunteers but it is also expensive and time consuming to collect samples for daily assays. We analysed the practical aspects of using a microelectronic personal fertility monitor as an alternative
to the laboratory based hormone assays, in a study that investigated the mechanism of action of an emergency contraceptive (Chapter 5).

Withdrawal of progesterone is the physiological trigger for menstruation. In 1988, Johannisson et al. suggested that the menstrual bleeding which follows the administration of mifepristone in luteal phase might be due to a direct effect on the capillary vessels of the endometrium. Further understanding of the mechanism by which mifepristone induces endometrial shedding may contribute in the future to the development of novel methods of contraceptives. To investigate this issue further, we evaluated the effects of mid-luteal phase administration of mifepristone on endometrial parameters (Chapter 6).

1.2 Progesterone

Progesterone is a natural steroid hormone that is produced by the corpus luteum in mammals. It is fundamental for the initiation, establishment and maintenance of pregnancy, whereas the cessation of progesterone secretion results in parturition, lactation and menstruation. Although the primary target tissues for the actions of progesterone are in the female reproductive tract, it also acts on the mammary gland (lobular-alveolar development in preparation for milk secretion and suppression of milk protein synthesis prior to parturition), on the central nervous system (mediating sexual behaviour in rodents) (Mani et al. 1994 a&b) and, on the thymus (inducing thymic involution) (Tibbetts et al. 1999). Corner and Allen were the first to isolate progesterone from the pig corpus luteum. In 1929, they demonstrated that when injected to overiectomised animals, progesterone can induce endometrial morphological changes similar to those of the early pregnancy, and has an anti-abortifacient effect (Corner & Allen 1929). Subsequently, in 1934, several independently working groups reported isolation of the crystallised progesterone (Allen & Wintersteiner 1934; Butenandt et al. 1934; Hartmann & Wettstein 1934). In the following year, Butenandt succeeded in
synthesising progesterone using cholesterol, and by 1938 several other synthetic progestins were commercially available.

Since then, several other important discoveries have contributed to our current understanding of this important hormone. These include the radio-immuno assaying techniques that accurately quantify progesterone by Lieberman et al. (1959), the identification of the uterine progesterone receptor by Milgrom & Baulieu (1970), the demonstration of the role of progesterone in the establishment of and maintenance of pregnancy by Csapo & Pulkkinen, (1978) and, the confirmation of this pivotal role of progesterone in fertility with the progesterone receptor knock-out mouse (PRKO) by Coneelly et al. in 2001. The pathways of progesterone action in target tissues are still not fully understood and, in many regards, distinctions remain to be made between the direct effects of progesterone and indirect consequences of progesterone regulation. Furthermore, progesterone mediates its effects through the progesterone receptor (PR) and PR is induced by oestrogen in most target tissues. Therefore, mapping out the progesterone specific effects, as distinct from those of oestrogen, is a complicated task.

1.2.1 Structure and metabolism

![Figure 1.1 Progesterone](image-url)
Progesterone is a C21 steroid (Figure 1.1) that belongs to the progestin class of ovarian hormones on the basis of its biological function. It is converted from cholesterol in the follicular cells. The delivery of cholesterol to the inner mitochondrial membrane is the rate-limiting step in progesterone synthesis. In corpus luteum, the steroidalogenic acute regulatory protein (StAR) mediates this process (Review by Strauss 2000 references therein). Cytochrome P-450 enzyme system that is responsible for catalysing the cleavage of C-20, 22 bond is located in the inner mitochondrial membranes and pregnenolone (C22) is formed as illustrated in Figure 1.2 (Farkash et al. 1986; Lieberman & Lin 2001). Thereafter, the 3β-hydroxysteroid dehydrogenase: 5-4 isomerase enzyme complex functions as a single entity to further convert pregnenolone to progesterone. Finally, the principle catabolite of progesterone, pregnanediol, is formed in the liver (Swart et al. 1993) and is eliminated by the kidney.

**Figure 1.2** Progesterone synthesis from cholesterol
1.2.2 Progesterone Receptor (PR)

**Figure 1.3**  The structural organization of the Progesterone Receptor

This figure illustrates the human PR cDNA, PR<sub>A</sub> and PR<sub>B</sub> proteins.

TAF-1 & 3: Amino terminal containing a constitutional ligand-independent trans-activation function (TAF-1 and TAF-3); DBD: central DNA-binding domain; H: the hinge region; LBD: the ligand-binding domain. The arrows indicate the translation start site for each receptor subtype. PR<sub>B</sub> is 933 amino acids in length and PR<sub>A</sub> lacks the N-terminal 164 amino acids.

The gestation-promoting properties of progesterone are exerted via interacting with specific nuclear receptor proteins that are induced by oestrogen. Conversely, in most target tissues, the expression of PR is decreased by its own ligand, progesterone. Progesterone displays lower affinity to PR, when compared with oestrogen binding to oestrogen receptor (ER). At high concentrations glucocorticoids can bind to PR (Walters & Clark 1977), whilst progesterone too can bind to both the glucocorticoid receptor (GR) and the androgen receptor (AR) at similar high concentrations (Lippman et al. 1977; Rousseau et al. 1973). Universally, the cellular uptake of progesterone appears to have no impediments thus, is rapid. The retention of the hormone in target cells seems to be attributable to the presence of PR (Clark & Sutherland 1979).
1.2.2.1 Structure of the Progesterone Receptor

PR belongs to a large steroid receptor super family of ligand-activated nuclear transcription regulators. They are characterised by organisation into specific functional domains that are relatively conserved between species and family members. The "consensus" PR consists of 4 different domains (Kumar et al. 1987; Evans 1988; Carson-Jurica et al. 1990; Figure 1.3).

1. The N-terminal domain contains a constitutional ligand-independent transactivation function (TAF-1 and TAF-3), which specifies the promoter of the target gene activation (Tora et al. 1988; Sartorius et al. 1994). This region appears to differ between species (Conneeley & Lydon, 2000a).

2. The highly conserved central DNA-binding domain (DBD) interacts with specific DNA sequences -the hormone regulatory elements (HRE) - in the target genes and it also contributes to receptor dimerisation (Freedman et al. 1992; Peterson et al. 2000).

3. Sandwiched between the DBD and the ligand-binding domain (LBD) is the hinge region.

4. The LBD at the carboxy-terminal contains a second ligand-dependent transactivation function (TAF-2). In addition to the obvious ligand binding activity, this region is important for receptor dimerization, interaction with heat shock proteins (hsp), and inter- and intra-molecular silencing (Webster et al. 1988; Fawell et al. 1990; Vegeto et al. 1992; Lanz & Rusconi 1994; Conneeley et al. 2000b).

Since the discovery of PR (Laumas & Farooq 1966; O'Malley et al. 1969; Sherman et al. 1970; Milgrom & Baulieu 1970), two natural forms of the human PR (PR_A and PR_B) have been characterised, and extensively studied. Both iso-forms bind progesterone and, are derived from transcripts initiated from two distinct promoters within the same PR gene (Kastner et al. 1990). Their functional properties differ in a cell type, promoter, or ligand specific manner (Tora et al. 1988; Chalbos et al. 1994). Thus, existences of these
PR isoforms may add to the complexity of progesterone action. Furthermore, the tissue distribution of these PR isoforms, and their ratio in reproductive tissues is influenced by developmental status (Shyamala et al. 1990), hormonal status (Duffy et al. 1997; Mangal et al. 1997) and malignant transformations (Graham et al. 1996). The full length PR\(_B\) (114 kDa), contains an additional N-terminal transactivation region, hence in the presence of progesterone, has a greater transcriptional efficacy than the truncated PR\(_A\) version. When expressed in the same cell in equi-molar ratios, with progesterone binding PR\(_A\) & PR\(_B\) may dimerise and bind to DNA as PR\(_A\):PR\(_A\), PR\(_A\):PR\(_B\), or PR\(_B\):PR\(_B\) (Conneely et al. 2001). Although usually the progesterone activated PR\(_B\) homo-dimers bind positively acting PREs to induce gene transcription, activated PR\(_A\) homo and hetero dimers appear to repress transcription of specific genes. PR\(_A\) (94 kDa) exhibit a trans-dominant negative activity, towards other steroid receptors including PR\(_B\), ER, GR and AR (Vegeto et al. 1993; McDonnell et al. 1994 a&b; Saatcioglu et al. 1994). Since PR\(_A\) can act as a dominant repressor of PR\(_B\) (Vegeto et al. 1993; Saatcioglu et al. 1994), the ratio of PR\(_A\) to PR\(_B\) expression may determine the progestin responsiveness in a particular cell type.

Production of PR subtype knock-out mice by Conneely et al. has provided the opportunity to test their functional roles at least in the rodent model (Conneely et al. 2001). The PR null mouse (PR\(_A\) & PR\(_B\) knock-out) showed several reproductive abnormalities including failure of ovulation, uterine hyperplasia and inflammation, defective implantation, lack of sexual behaviour in response to progesterone, abnormal morphology of the mammary gland and defective thymic imuno-adaptation to pregnancy (Lydon et al. 1995). The suppressive function for P in uterus appears to be due to PR\(_A\) while both the isoforms are involved in ovulatory process (Conneely et al. 2001). The epithelial hyperplasia seen in PR\(_A\) knockout mice may suggest that PR\(_B\) facilitates endometrial hyperplasia while PR\(_A\) inhibits it, and this may have obvious relevance to pharmacological management of endometrial dysplasias.
1.2.2.2 Progesterone binding and cellular mechanism of action of the progesterone receptor

Free PR forms an inactive, non-DNA-binding hetero-oligomeric 8S complex, which upon agonist binding dissociates in to homo-dimers with DNA binding capacity. 42 C-terminal amino acids of the LBD region are mandatory for progesterone (agonist) binding (Carson-Jurica et al. 1990; Vegeto et al. 1992). The 8S complex includes receptor-associated proteins such as heat shock protein hsp90 and immunophilin FKBP59/HBI. These proteins play a role in stabilising the hetero-oligomeric receptor complex (Baulieu et al. 1989 a, b&c; Lebeau et al. 1993). Furthermore, the multipoint hsp90 binding to PR involves both LBD and DBD thus, obliterates the DNA binding to the DBD (Bourgeois et al. 1984; Baulieu & Catelli 1989b). Progesterone binding induces trans-conformation (Allan et al. 1992) that initiates dissociation of the heat-shock proteins from the non-DNA binding receptor complex, homo-dimerisation (Tsai et al. 1988), increased receptor phosphorylation (Edwards et al. 1993; Weigle et al. 1993 & 1994) thus activating the receptor.

1.2.2.3 Mechanisms of action of progesterone through classic hormone regulatory element

The activated PR binds to the progesterone regulatory element (PRE) of a target gene, a specific DNA sequence that is located in the promoter, upstream of the coding sequence. Consensus HREs consist of palindromic hexanucleotide motifs separated by three-nucleotide spacers (Beato et al. 1989 & 1996). Binding of the progesterone-PR complex to the two halves of the same palindromic PRE results in gene transcription via the action of RNA polymerase. How exactly the activated PR couples with the general transcriptional machinery is not well understood. Nevertheless, recent data on PR and other steroid receptors suggest a possible involvement of specific co-activators, co-repressors, specific-DNA binding transcription factors and, general transcriptional factors. It has been shown that co-activators (e.g. SRC family, CBP/p300, E3 ubiquitin-protein ligases, L7/SPA, HMG-1/2 and, SRA) are recruited by the progesterone-bound
PR and, they enhance receptor-dependent trans-activation (Rowan 2000 & references therein). Agonist-bound PR may also induce remodelling of the chromatin structure thereby facilitating the DBD and PRE interaction and recruiting transcription factors (Truss et al. 1993 & 1994).

The two transcription activation function areas (in the DNA binding domain & in the LBD) within the PR further regulate the initiation of gene transcription (Cadepond et al. 1997). Activity of TAF-1 depends on receptor and promoter types and cell-specific factors (Meyer et al. 1990; Vegeto et al. 1993), while only agonists are able to activate the TAF-2 in LBD, possibly by opposing an inhibitory activity that is located at the C-terminus of the PR (Vegeto et al. 1992). In addition, upon DNA binding PR undergoes N-terminal mediated changes outside the DBD (Bain et al. 2000).

PR, in parallel with the other members of the steroid receptor super family, is a phosphoprotein and, agonist induced phosphorylation of the PR is associated with enhanced specific-DNA binding which may consequently enhance the transcriptional activity (Beck et al. 1992; Edwards et al. 1993; Weigel et al. 1993 & 1994).

In addition to the above described progesterone induced transcription activation, recent data has proposed alternative ligand independent pathways of PR activation, which involve altered cellular conditions due to increased intracellular kinase activity, or stimulating intracellular phosphorylation (Conneely et al. 2000 and references therein).

Therefore, following progesterone binding, the PR undergoes a conformational change and initiates a number of progesterone-dependent transcription processes that mediate the genomic response to the hormone.
1.2.2.4 Novel mechanisms of action through PR interaction with alternative sequence-specific transcription factors.

Interestingly, it has been shown that progesterone-PR complex is able to either repress or enhance transcription of different proteins that depended on the same transcription factor. In normal mammary epithelial cells, under the same cellular conditions but different promoter context, PR is able either repress (β-casein) or enhance (3β-HSD) Stat5 (signal transducer and activator of transcription)-mediated gene transcription. The 3β-HSD promoter region only contains a Stat5 response element and, progesterone induced 3β-HSD gene transcription appears to be Stat5 dependent. In the same cell type, the liganded PR suppressed the Stat5 dependent β-casein transcription. It has been proposed that both PR and Stat5 compete for a composite binding site in the β-casein promoter, and that occupancy by the liganded PR prevents Stat5 transactivation function (Edwards et al. 2000).

In breast cancer cells, PR activates the c-Src tyrosine kinase in a rapid, progesterone dependent manner and this action is independent of gene transcription but involves the prolin-rich motif of the N terminus of PR (Edwards 2002).

1.2.2.5 Non-genomic actions of progesterone

There is now compelling evidence for the existence of progesterone effects that cannot be explained by the classic model of steroid-target cell interaction. These effects tend to be incompatible with mRNA and protein synthesis (rapid and can be observed in cells that do not have transcriptional ability e.g. spermatozoa; and transcription inhibitors are unable to block these effects) and, may not involve PR or progesterone entering the cell, yet are highly specific and may involve surface membrane binding sites (Revelli et al. 1998). Data exist on non-genomic actions of progesterone in granulosa cells (Machelon et al. 1996), in amphibian oocyte maturation (Sadler et al. 1985; Revelli et al. 1998 and references therein), and in spermatozoa (Bray 1999). However, the importance of these
non-genomic responses to progesterone in human female reproduction remains to be determined.

1.2.3 Endometrial steroid receptors

Immuno-histochemical studies employing monoclonal antibodies have made it possible to demonstrate the nuclear location of the steroid receptors in different endometrial compartments (McCarty et al. 1985; Press et al. 1988). During the proliferative phase, PR concentration increases in both epithelial and stromal compartments. Although, this remains high in the early luteal phase (predominantly in the glands), there is a decline in the mid- to late secretory phase (Garcia et al. 1988; Lessey et al. 1988; Berthois et al. 1991). The observed decrease in the PR expression during the latter part of the cycle is pronounced in the glandular epithelium, when compared to the stromal compartment (Bergeron et al. 1988; Lessey et al. 1988; Press et al. 1988; Snijders et al. 1992; Fung et al. 1994; Moutsatsou et al. 1997). Furthermore, within the stromal compartment, PR showed a specific localisation in the perivascular regions (Critchley et al. 1998a).

Several groups have shown that in the human endometrium, the PR expression varies with the menstrual cycle phase, and reach a maximum during the early luteal phase (Janne et al. 1975; Bergeron et al. 1988; Garcia et al. 1988; Lessy et al. 1988; Press et al. 1988; Snijders et al. 1992; Fung et al. 1994; Zeimet et al. 1994; Moutsatsou et al. 1997). Oestrogen and progesterone sequentially regulate endometrial PR expression. Oestrogen induces PR protein synthesis by mediating an increase in PR mRNA throughout the proliferative phase (Clarke 1990) while progesterone down regulates PR at transcriptional and posttranscriptional levels throughout the secretory phase (Chauchereau et al. 1992). Although the smaller A-receptor remains the phenotypically dominant iso-form in the endometrium, the PR$_A$: PR$_B$ expression ratio is increased in the peri-menstrual period of the cycle (Tung et al. 1993; Mangal et al. 1997). The transdominant negative effect of PR$_A$ may produce the observed inhibitory effects of progesterone and antiprogesterones on ER expression. Furthermore, in mid-luteal phase endometrial glands, PR$_B$ may be the predominant PR subtype (Mote et al. 1999).
In the secretory endometrium, AR expression had been observed predominantly in the stroma with barely detectable AR immunoreactivity in the glandular epithelium (Slayden et al. 2001). Immediately before menstruation in the late secretory phase, a decrease in stromal AR immuno-staining had been reported (Slayden et al. 2001). In contrast, an up-regulation was observed in glands as well as in stroma, following the early luteal phase administration of mifepristone (Slayden et al. 2001).

1.2.4 Myometrial PR
Recent evidence suggests that the non-pregnant uterus acts as a peristaltic pump during the menstrual cycle with directed sperm transport being one of the main functions (Lyons et al. 1991; Kunz et al. 1996; Leyendecker et al. 1996). Noe and colleagues demonstrated a cyclical pattern of ER and PR expression in the subepithelial myometrial cells, which parallels that of the endometrium. They went on to suggest that progesterone might be involved in the very early processes of reproduction, by regulating the cyclical uterine peristalsis (which is confined to the endometrium and sub-endometrial myometrium) that assists rapid and sustained sperm transport, in addition to the action on the endometrium. The outer myometrial layers did not exhibit a cyclical pattern of PR expression, but the immuno staining remained strong throughout the whole cycle. This cell-specific differential receptor regulation might be functionally significant in suppressing the action of progesterone in the subendometrial myometrium (supressing the cyclical uterine peristalsis) in the presence of high circulating concentrations of this hormone required for continuing action in the outer myometrial layers (maintaining myofibrils at a quiescent state) (Noe et al. 1999).

1.2.5 Progesterone effects on the uterus
Progesterone acts upon the endometrium to initiate either directly or indirectly appropriate changes in structure responsible for the required functional end points, implantation, regeneration and repair. If fertilisation were to occur, the high circulating levels of progesterone that result, facilitate implantation, stimulate uterine growth and
oppose the action of factors involved in myometrial contractility. Conversely, at the end of an infertile cycle, withdrawal of progesterone will induce menstruation. Moreover, the anti-proliferative / anti-oestrogenic effects of progesterone in the endometrium have been extensively studied; They include the down-regulation of ER (Hsueh et al. 1970; Katzenellenbogen et al. 1980), the increased metabolism of oestradiol to less active oestrone (Tseng & Gurpide 1975), and the decrease of oestrogen-induced proteins and proto-oncogenes (Bhakoo et al. 1977; Kirkland et al. 1992; Elger et al. 2000).

1.3 Levonorgestrel

LNG (systemic name 17β hydroxy- 17α ethinyl- 13β ethyl- 4 gonen- 3 – one) is the biologically active dextro-rotatory isomer of the synthetic progestin norgestrel, and has more potent progestational and anti-ovulatory action than the naturally occurring progesterone (Khan et al. 1983; Stanczyk et al. 1994). Various formulations of LNG are currently being used clinically, including; (i) in combination with ethinyloestradiol for the combined oral contraceptive pill or for Yuzpe regimen of post coital contraceptive, (ii) in combination with ‘natural’ oestrogens for hormone replacement therapy, (iii) on its own as LNG only post coital contraception and as LNG releasing IUCD, intravaginal rings, and subcutaneous implants. Pharmacokinetics of LNG depends on the particular formulation.

![Structure of Levonorgestrel](image)
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1.3.1. Pharmacokinetics of LNG

Since LNG is not metabolised through the entero-hepatic circulation (Fotherby et al. 1994), the bio-availability of the oral dose is high. When administered orally, it is rapidly absorbed, and achieves the maximum serum concentration (t max) within 2 hours (Goebelsman et al. 1986; He et al. 1990; Kuhnz et al. 1992; Kook et al. 2002). In common with the other synthetic gestogens (Fotherby 1983) LNG shows a large inter- and intra individual variability in the terminal half-life of elimination (mean values ranging from 8 - 26 hours) (Weiner et al. 1976; Goebelsman et al. 1986; Back et al. 1987; Fotherby et al. 1990; Tremblay et al. 2001; Kook et al. 2002). The importance of hormone sensitive sex hormone binding globulin (SHBG) in LNG pharmacokinetics has been reported by several investigators (Victor et al. 1976 & 1977; Affandi et al. 1987). In blood, LNG binds to both albumin, and (with high affinity) to SHBG. LNG dissociates slowly from the stable LNG-SHBG complex (Juchem et al. 1990; Fotherby et al. 1994), and only the unbound fraction in serum (less than 2% of the total drug) is responsible for the biological action. On the other hand, LNG directly affects serum SHBG, and a single dose of 0.75 mg LNG was able to reduce the circulating SHBG by 25% (He 1990). However, this action is reversed by the concomitantly administered ethinyloestradiol, thereby affecting the pharmacokinetics of LNG (Fotherby et al. 1990). The studies evaluating the pharmacokinetics of LNG had small numbers, and did not determine the SHBG level at the time of administration of LNG. These deficiencies may explain the variability seen in the pharmacokinetic profile of LNG since the range of SHBG levels among healthy women is reported to be wide (Fotherby et al. 1995). The metabolites of LNG are present in the circulation at greater concentrations than LNG for a longer time period (Warren et al. 1974). If biologically active, they may sustain the progestagenic effects for a much longer period than expected. Once more, this possibility is yet to be elucidated. Therefore, the complex pharmacokinetics of LNG makes its correlation to the pharmacodynamic activity difficult. However, recent studies have shown that following 0.75mg of LNG taken twice 24 hours apart, the plasma LNG level remains above 1 nmol/L for approximately 4.5 days (Tremberly et al. 2001). This is of particular
importance, as it is the minimal plasma level of LNG required to inhibit ovulation (Nilsson et al. 1980).

1.4 Mifepristone

Agents that antagonise the effects of progesterone were actively sought even prior to the present day understanding of the cellular mechanism of action of hormones. Although unsuccessful in finding a synthetic anti-progestagen, Pincus was the first to postulate the potential value of a compound with anti-gestagenic activity in fertility control (Pincus 1965). Since then, many others have made several important contributions (Sherman et al. 1970; Belanger et al. 1981) that finally led to the discovery of mifepristone (RU 38486, subsequently abbreviated to RU 486), which was the first potent anti-gestagen to be synthesised (Philibert et al. 1981). Two decades have passed since and, unfortunately the political controversy surrounding mifepristone as an “abortion pill” has hindered its many other potential therapeutic uses being put to practise.

Mifepristone exerts its potent antiprogesterone and antiglucocorticoid action in humans by binding to the intracellular receptors of the antagonised hormones. Despite the close structural relationship between the steroid receptors, mifepristone does not bind to ER or the mineralocorticoid receptor (MR) yet demonstrates high affinity for PR, GR, and also (somewhat lower affinity) for AR. Upon binding mifepristone maintains the PR in a biologically inert conformation that precludes progesterone binding (Evans et al. 1988; Baulieu 1989) and itself does not activates the PR.
1.4.1. Structure of mifepristone

Figure 1.5 Mifepristone

Mifepristone is a 19-norsteroid that lacks the C19-methyl group of natural progesterone and of glucocorticoids. The chemical structure of mifepristone shows two important distinctive differences from the steroidal skeleton, a 4-dimethyl amino-phenyl group substituted at the 11β position, and a 1-propynyl-chain substitution at the 17α position (Figure 1.5). The higher binding affinity of mifepristone to PR is thought to be due to both these variations, while the size and positioning of the former determines the anti-progesterone action (Evans et al. 1988; Baulieu 1989; Garcia et al. 1992; Spitz et al. 1993). The 17α-propynyl side chain appears to confer pure-glucocorticoid activity, and various modifications at C-17 position of the RU 486 structure resulted in compounds with relatively lower anti-glucocorticoid activity. However, to date no pure progesterone antagonist that is totally devoid of glucocorticoid activity has been described.
1.4.2 Pharmacokinetics of mifepristone

Mifepristone is usually administered orally as a single or multiple doses and the dosage varies from 0.5 mg to 800 mg daily in various studies. The oral dose is absorbed rapidly (70%) and is subject to the first-pass metabolism in the liver. Bio-availability after the oral dose in humans (40%) does not appear to be affected by the pregnant status or the ethnicity but it is reduced in some animal species (Spitz et al. 1993, Sarkar 2002). Mifepristone is metabolised in the liver and is excreted mainly in urine and faeces as biologically active demethylated and hydroxylated metabolites.

1.4.2.1 Receptor binding and ligand-induced trans-conformation

Mifepristone is a potent antagonist of progesterone action in vivo, however the mechanism of this antagonism is poorly understood. Mifepristone binds to PR with a relative binding capacity five times that of progesterone (K of dissociation <1 nM) and does not activate the receptor (Philibert 1984; Skafar et al. 1991). The affinity of mifepristone for the GR is also of same order of magnitude as that for PR. The magnitude of the anti-glucocorticoid activity however, is influenced by the phase of circadian rhythm and the negative pituitary feed-back of cortisol secretion and, requires a higher dose than that which has anti-progesterone activity (Healy et al. 1983 & 1985; Gaillard et al. 1984; Bertagna et al. 1984).

The PR of most species including humans contains a large hydrophobic pocket that can accommodate the equally hydrophobic 11β substitution of mifepristone (Teutsch et al. 1985). Mifepristone appears to require a set of N-terminal region amino acids of LBD on the PR that overlap with the progesterone binding, but are non-co- incidental. A monoclonal-antibody to a 14 amino acid sequence in the C-terminal of LBD only blocks progesterone binding, but not mifepristone (Weigel et al. 1992). Point mutations have been identified in the LBD that distinctly affect binding of either mifepristone {at Gly722 of the human PR (Benhamou et al. 1992)} or, progesterone {Cys575 of the chicken PR (Baulieu 1993)} but not the other; and a C-terminal mutant PR has been
shown to bind only mifepristone, but not progestin (Vegeto et al. 1992). The qualitative property of hormone antagonism however, does not appear to equate to mere binding ability. Although mifepristone does not bind to chicken PR (cPR), a change of a single amino acid in the N-terminal region of cPR LBD (Cys 575 to Gly) allows binding of both mifepristone and RU 39115 (RU 39115 is an antagonist of the human PR and, lacks the N-dimethyl substituent of mifepristone). Upon this binding however, the antiprogesterone activity is only observed with mifepristone. RU 39115 on the other hand, exhibits agonist activity in the mutant receptor (Gronemeyer et al. 1992; Baulieu 1993). Therefore, the overall structure of the LBD and the antagonist and, the TAF-2 function may influence the biological activity of a steroid hormone agonist or antagonist.

Mifepristone competes with progesterone for binding to PR and promotes many of the normal activation steps elicited by progesterone, including dissociation of the heat-shock proteins, dimerisation, and acquisition of high affinity for target DNA binding (Baulieu et al. 1988; El-Ashry et al. 1989; DeMarzo et al. 1992; Gass et al. 1992; Beck et al. 1993a). Although mifepristone-PR complex has higher affinity for PRE than that of progesterone-PR complex, upon binding to the PRE only the latter and not the former, activates the transcriptional machinery. Therefore, mifepristone is defined as a type II antagonist (other antiprogestagenic substances which form complexes with PR but are unable to bind to PRE (e.g. ZK 98299) are defined as type I antagonists) that appears to recapitulate the early steps of agonist-activation of receptor yet fails to activate a transcriptional response. Our understanding of the molecular mechanisms of this receptor inactivation is still far from complete. Nevertheless, the existing evidence is as follows. Mifepristone-PR complexes may form inactive, mixed ligand-dimer heterodimers with progesterone-PR complexes, while actively competing with them for PRE binding sites (Edwards et al. 2000); mifepristone induces ‘an inappropriate’ structural conformation at the extreme carboxy-terminus of the LBD, which is distinct from that induced by progesterone (Allan et al. 1992; Weigle et al. 1992); This altered conformation can prevent both the agonist-dependent TAF-2 activity and the binding of
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PR co-activators that enhance receptor-dependent transcription such as SRC (Edwards et al. 1999 & 2000); Mifepristone may also promote recruitment of inappropriate co-activators that are not selected by progesterone such as L7/SPA (Jackson et al. 1997); Additionally, it may actively recruit PR co-repressors and the co-activator and co-repressor concentration ratios may determine the antagonist or partial-agonist activity of mifepristone (McDonnell et al. 1992; Jackson et al. 1997; Smith et al. 1997).

In particular tissues, mifepristone may behave as a progesterone agonist. In the absence of progesterone, mifepristone exhibits a progesterone-like anti-oestrogenic effect in an oestrogen-primed endometrium (Wolf et al. 1989; Van Uem et al. 1989). However, if progesterone was present mifepristone acts as a pure antagonist. The repressor role of PR\textsubscript{A} on ER has been suggested as the explanation for this anti-oestrogenic effects of mifepristone (see Cadefond 1997 and references therein). Recently, Brenner et al. suggested that this non-competitive anti-oestrogenic effect of mifepristone seen on rhesus macaque endometrium is mediated via inducing AR expression (Brenner et al. 2002). Similar to progesterone, mifepristone inhibits prolactin Stat5-mediated induction of β-casein Luciferase receptor in the mammary epithelial cells. It has been postulated that this is due to the occupancy of the composite DNA-binding element located in the β-casein promoter by the mifepristone-PR complex which precludes the Stat5 binding, thereby suppressing Stat5 trans-activation function (Edwards et al. 2000). In vitro studies have exposed conditional agonist activity similar to other type II antagonists with mifepristone. Alteration of cellular conditions by either activation of protein kinase A or stimulation of cAMP signalling pathway resulted in antagonist-to-agonist switch in mifepristone activity in vitro (Beck et al. 1993b; Nordeen et al. 1995). It is of further interest that the weak partial agonist activity is associated with only mifepristone bound PR\textsubscript{B} but not PR\textsubscript{A} (Leonhardt & Edwards 2002).

In summary, the structure of mifepristone and of the LBD combine ultimately to direct the trans-conformation of PR, and DNA binding; but do not activate the TAF-2 or
induce transcription and thus, mifepristone acts as an antagonist. The production of a conformational change in the PR distinct from that induced by progestins is a central property of mifepristone.

1.4.3. Toxicology

1.4.3.1 Short-term administration

A single oral dose of mifepristone is eliminated within 6-7 days of administration (Sarkar 2000). The effects of short-term administration of mifepristone have been well characterised and, appear to be safe. Administering 200 mgs of mifepristone daily for 8 days to healthy men resulted in a hormonally detectable antiglucocorticoid state and a reversible cortisol overproduction with preservation of adrenocortical and pituitary reserves. Importantly, no concurrent clinical symptoms of peripheral cortisol deprivation were observed with these effects (Bertagna et al. 1994). Others have shown that the disinhibition of the pituitary-adrenal axis is only observed during the morning hours of the circadian rhythm, thus lowering the dosage and changing the time of administration may be important to minimize the side effects (Gaillard et al. 1984). One study reported that mifepristone at high doses (10 mg/Kg taken daily for a week) induced a diffuse maculo-papular erythematous cutaneous eruption within 2 weeks of discontinuation of the drug in the majority (73%) of the healthy male volunteers. Histologically, this generalised exanthem showed a diffuse dermal peri-vascular lymphocytic infiltration that resolved spontaneously (Laue et al. 1990). However, none of the affected subjects demonstrated an increase in the eosinophil count, which does not support the possibility of drug hypersensitivity or glucocorticoid deficiency being responsible for the skin reaction. Moreover, after taking even higher doses (20 mg/kg) for longer periods, others have not developed similar complications (Chrousos et al. 1988).
1.4.3.3  **Chronic administration**

Most novel therapeutic indications of mifepristone require long-term administration (e.g. breast cancer, endometriosis) and, its antiglucocorticoid action has raised the issue of safety with chronic exposure to the drug. Prolonged administration of daily low doses was not associated with a cumulative increase in plasma concentration of the drug (Heikinheimo et al. 1987 & 1989; Sarkar 2002). At doses above 200 mg a day, clearance of mifepristone from the body appears to reach a limiting value and this may produce elevated plasma levels over a long duration (Sarkar 2002). In a small proportion of volunteers, when high doses of mifepristone was administered continuously for more than several days, symptomatic glucocorticoid deficiency has been observed. Chronic exposure of 200 mgs a day was associated with symptoms of cortisol deprivation that were severe enough to be treated with concomitant prednisolone (Lamberts et al. 1991).

Lambert et al. postulated that mifepristone activated and reset the hypothalamo-pituitary-adrenal axis at a higher level. However, this observation has not been reported by others, using a similar regimen for as long as 15 months for the treatment of unresectable meningiomas (Heikinheimo et al. 2000). In pre-menopausal women, a similar regimen induced amenorrhoea, or metrorrhagia (in one case with endometrial hyperplasia) (Grunberg et al. 1993). The main concern with this chronic dose however, was the reported increase in oestrogen (as a consequence of the compensatory overproduction of androgen), which might limit the use of mifepristone in treating oestrogen dependent cancer. Conversely, recent data, which demonstrated a low oestrogen level with no discernible change in SHBG after chronic 200 mg daily dose, suggest that enhanced systemic oestrogen effects are unlikely during long-term mifepristone treatment (Heikinheimo et al. 2000).

Studies using long-term daily doses of mifepristone up to 10 mg/kg, or using once-off doses of up to 25 mg/kg, did not report any other adverse events. Since the doses required for most potential therapeutic applications of mifepristone are well within these dose limits, the safety aspect of mifepristone appears to be reassuring.
1.4.3.2 **Teratogenicity**

The use of mifepristone in the induction of abortion and as a contraceptive agent and the fact that it traverses the placental barrier (Hill *et al.* 1991), raises concerns about the possibility of teratogenic effects in infants who are exposed to mifepristone during early gestation (Doherty *et al.* 1992). Unfortunately, the available data on this possibility are rather limited. In rabbits, even at sub-abortive doses mifepristone induced foetal abnormalities (Yost *et al.* 1986; Wolf *et al.* 1990). However, monkey embryos did not show teratogenicity when exposed to mifepristone either before implantation (10^{-7} M) or immediately post-implantation (50 mg/day for 7 days) (Wolf *et al.* 1990). Henrion *et al.* in 1989 reported a single case in which administration of mifepristone in the first trimester was associated with second trimester fetal-malformation (Henrion *et al.* 1989). In a more recent report, Sitruk-Ware *et al.* reviewed 71 cases of continuing pregnancy following failed early medical termination (occurred between 1987-1998) and reported only one case of fetal abnormality after the use of mifepristone alone. There were 7 further cases of fetal abnormality following treatment of mifepristone in combination with prostaglandins (Sitruk-Ware *et al.* 1998). No other instances of possible teratogenicity had been reported despite the growing use of mifepristone in inducing medical abortion around the world. Conversely, there have been reports of human fetuses that have been exposed to mifepristone but not subsequently aborted, developing normally (Lim *et al.* 1990).

1.5 **Menstrual cycle**

Throughout a woman’s reproductive life, the dynamic relationship between the pituitary and ovarian hormones allows the cyclical sequence of events, which commences with folliculogenesis and proceeds to extrusion of a mature oocyte and transformation of the follicular granulosa cells into a corpus luteum which eventually undergoes luteolysis, thus terminating an infertile cycle.
1.5.1 Progesterone regulation of the hypothalamo-pituitary-ovarian axis

The feedback mechanism that exists between the ovary and the hypothalamo-pituitary unit is responsible for the normal cyclical ovarian activity (Knobil et al. 1980). The anterior pituitary produces LH and Follicle Stimulating Hormone (FSH) in a pulsatile fashion in response to the hypothalamic neuropeptide; gonadotrophin releasing hormone (GnRH). The ovarian hormones oestrogen, progesterone and inhibin also influence this regulation, and conversely, LH and FSH directly regulate these hormones. Normal LH secretion (in a pulsatile manner) and ovulation are dependent on the release of GnRH pulses that happen at a critical frequency and amplitude (Dierschke et al. 1970; Leyendecker et al. 1979). The LH pulses increase in frequency immediately before ovulation, then following ovulation slow down while increasing their amplitude in the first half of the luteal phase (Backstrom et al. 1982; Wildt et al. 1981; Filicori et al. 1986). It has been shown that the pre-ovulatory surge of LH triggers ovulation by initiating a series of changes in the dominant follicle causing the rupture and the subsequent release of the ovum. Furthermore, LH can stimulate a transient increase in PR mRNA expression in pre-ovulatory follicular granulosa cells suggesting an LH induced progesterone responsiveness in those cells (Natraj et al. 1993; Park-Starge et al. 1994). Normal women have a pre-ovulatory rise in progesterone preceding the initiation of the mid-cycle LH surge (Hoff et al. 1983). This is thought to facilitate oestrogen in releasing the LH surge (Odell et al. 1968; Chang & Jaffe 1978; March et al. 1979 & 1981; Wildt et al. 1981; Lui & Yen 1983; Mahesh & Muldoon 1987; Permezel et al. 1989; Leyendecker et al. 1990) and may also play an important role in the contemporaneous discharge of the gonadotrophins (Aono et al. 1976).

Presently available evidence for the effects of progesterone on the neuro-endocrine axis during the luteal phase is questionable, since studies using antiprogestosterone have not always produced the expected results (Herrman et al. 1982; Critchley et al. 1988; Garzo et al. 1988; Shoupe et al. 1990). After ovulation, the facilitatory role of progesterone in releasing LH reverses rapidly into one of an inhibitory nature, (Leyendecker et al. 1972;
Drouin & Labrie 1981; Araki et al. 1985) changing the high frequency, low amplitude LH pulses of the follicular phase to pulses of low frequency and high amplitude (Hoff et al. 1983; Soules et al. 1984). The pulse frequency of LH declines progressively during the luteal phase and correlates with cumulative progesterone exposure (Filicori et al. 1984; Soules et al. 1984). Moreover, exogenous progesterone has been shown to decrease LH pulse amplitude (Soules et al. 1984). Endogenous opioids appear to influence LH pulsatility via inhibiting GnRH secretion (Rasmussen et al. 1983). Since the maximum suppression occurs when high progesterone levels are seen (Soules et al. 1984; Shoupe et al. 1985) and this effect is reversed by naloxone infusion in the luteal phase (Quigley & Yen 1980), it was thought that progesterone was important in modulating the endogenous opioid mediated inhibitory process (Quigley et al. 1980; Shoupe et al. 1985). More complex actions of anti-gestagens that are not solely due to progesterone antagonism may explain the unexpectedly different results seen in studies using anti-progesterone (Herrman et al. 1982; Critchley et al. 1988; Garzo et al. 1988; Shoupe et al. 1990). Nevertheless, immunisation of the ewe against progesterone has consistently produced an increase in the LH pulse frequency supporting the inhibitory effect of progesterone on gonadotrophin secretion (Thomas et al. 1987).

1.5.2 Control of Ovarian cycle

In humans, folliculogenesis, ovulation and corpus luteum formation occur under pituitary gonadotrophic control.

1.5.2.1 Follicular phase

Various ovarian follicular compartments interact in a highly integrated manner to respond to cyclic gonadotrophic hormones by secreting sex steroids and producing a fertilizable, mature ovum. Progesterone and oestrogen levels remain low during the menses, and thereafter, in the follicular phase, the developing Graafian follicle produces increasing levels of oestradiol (Yoshinga et al. 1978). FSH stimulates the growth of the follicles beyond the stage of antrum formation (Baird 1987 & 1990 and references
therein). By the mid-proliferative phase of the cycle, FSH-inducible granulosa cells of the apparent single dominant follicle (≥10 mm diameter) produce increasing amounts of oestrogen by aromatising the androgens derived from LH-dependent theca cells (Yoshinga et al. 1978). Oestrogen and inhibin secretion from the enlarging preovulatory dominant follicle (usually one per menstrual cycle) suppresses (negative feed back) the pituitary FSH release, while inducing an LH surge, which initiates ovulatory process (Baird 1990 and references therein). Ovulation seems to occur approximately 36 hours after the onset of the LH surge (Seibel et al. 1982).

1.5.2.2 Control of Ovulation
The LH surge initiates a cascade of proteolytic events that control ovulation and, also induce PR gene expression in granulosa cells of the mature preovulatory follicles (Natraj et al. 1993). Furthermore, around the time of the mid-cycle LH surge in women, pre-ovulatory follicles appear to secrete increasing levels of progesterone (Zander et al. 1954; Hoff et al. 1983; Djahanbakhch et al. 1984). The presence of PR in most follicular cell types, failure of ovulation in PR null mice despite having mature preovulatory follicles (Suzuki et al. 1994; Lydon et al. 1995) and, the inability of the mature follicle to rupture in the absence of mid-cycle progesterone secretion (Schenken et al. 1986) suggest that the ovulatory process is regulated by progesterone. It is postulated that this regulatory pathway may involve relaxin, kallikrein, proteases (e.g. cathepsin L, ADAMTS-1), and plasminogen activator (Yki-Jarvinen et al. 1985; Too et al. 1984; Tanaka et al. 1992; Robker et al. 2000).

1.5.2.3 Luteal phase
The luteal phase of the cycle is dominated by progesterone arising from the corpus luteum. Conversely, formation of corpus luteum appears to be progesterone-dependent and, indeed PR is expressed in the corpus luteum despite high local progesterone concentrations (Aladin Chandrasekher et al. 1994). After ovulation, progesterone levels reach a peak by day 8 or 9 and, if implantation does not occur, progesterone declines by
day 10-12 (first gradually and then precipitously). In the latter part of the infertile cycle, the corpus luteum regresses, stops the release of progesterone and, consequently a decline is observed in the circulating levels. This withdrawal of progesterone causes the endometrial pseudo-decidua to degenerate, which results in menses. Luteal regression permits initiation of a subsequent cycle, by allowing the development of a fresh batch of follicles.

1.5.3 Effects of mifepristone on the hypothalamo-pituitary-ovarian axis and on the ovarian cycle

Mifepristone binds to PRs in the hypothalamus, the anterior pituitary and in the ovary as well as the uterus. It thereby has multiple effects on the hypothalamic-pituitary-ovarian axis and on the ovarian cycle depending on the time of the cycle and the dose used.

1.5.3.1 Follicular phase administration of mifepristone

During the early follicular phase except for a minimal decrease in oestradiol level, mifepristone (at a dose of 3 mg/kg) has no effects on the ovarian cycle (Stuenkel et al. 1990). However, later in the follicular phase, mifepristone arrests folliculogenesis and inhibits or delays ovulation, while decreasing oestradiol and LH levels during treatment (Shoupe et al. 1987; Permezel et al. 1989; Stuenkel et al. 1990). In vitro studies have shown mifepristone to inhibit LH secretion by cultured rat pituicytes (Wolf et al. 1989). Additionally the reduction of LH pulse amplitude seen after late follicular administration of mifepristone to women suggests a pituitary site of action (Permezel et al. 1989). Therefore, the inhibition of ovulation seen after administering high doses (> 50 mg) in the mid-late follicular phase of the cycle seems to be due to the suppression of the secretion of pituitary gonadotrophins (both LH and FSH) thereby arresting follicular growth (Luukkainen et al. 1988; Nieman 1993; Liu et al. 1987; Shoupe et al. 1987; Permezel et al. 1989). Afterwards, follicular recruitment can reinitiate once the treatment is discontinued and ovulation can occur approximately 2 weeks later (Liu et al. 1987; Shoupe et al. 1987).
The delay of the mid-cycle LH surge seen after continuous administration of mifepristone (1 mg daily, over 5-15 days after the dominant follicle selection) in the late follicular phase can be prevented by concomitantly administered exogenous progesterone (Batista et al. 1992a). The mechanism for this appears to be via inhibition of the positive feedback effect of oestrogens (Baird et al. 1995). It has been shown that daily administration of 2 mg of mifepristone does not affect the pulsatile pattern of LH secretion (Baird et al. 1995). Therefore, the impairment of oestrogen induced LH surge is not due to an effect of mifepristone at the hypothalamic level and nor is it due to a decreased responsiveness of the pituitary to exogenous GnRH. On the basis of these observations Baird et al. concluded that mifepristone at this dose (2 mg daily) interferes with the mid-cycle LH surge by a complex mechanism, possibly by directly reducing the sensitivity of pituitary gonadotroph to the positive feed-back effects of oestrogen.

It is important to note that the above studies reported blocking of the LH surge when multiple doses of mifepristone were administered continuously in the follicular phase. The lowest single dose, which will prevent the LH surge and ovulation consistently, is not known and once the LH surge is initiated mifepristone does not seem to be able to arrest ovulation (Liu et al. 1987; Marions et al. 2002; Brown, A. unpublished data through personal communication).

### 1.5.3.2 Luteal phase administration of mifepristone

In the mid-luteal phase a single dose of mifepristone (5 mg/kg) acutely increases both the amplitude and the frequency of LH pulses (Critchley & Baird 1988). After this initial effect, some authors have reported a decline in the secretion of total LH and in the pulse amplitude, but no discernible effect was seen on the pulse frequency (Garzo et al. 1988; Shoupe et al. 1990). It has therefore been suggested that this failure of an antiprogesterone to influence LH pulse frequency may be due to a lack of a direct hypothalamic effect. Although others have reported a decrease in LH pulse frequency after mifepristone, due to the low frequency of LH pulses detected, the validity of their
result is uncertain (Schaison et al. 1985). Low doses, 10 mg for example, do not affect LH levels (Greene et al. 1992). Therefore, during the mid-luteal phase, mifepristone may initially relieve a suppressive effect of progesterone on the frequency and amplitude of LH pulses, and thereafter it may have a direct pituitary action in decreasing the LH pulse amplitude. Moreover, naloxone did not reverse this mifepristone (a single dose of 100 mg mifepristone) induced decrease in the LH pulse amplitude (Shoupe et al. 1990) suggesting that the inhibitory hypothalamic effect of endogenous opioid peptides may not be fully dependent on progesterone as previously thought.

In the late luteal phase a single dose of 600 mg of mifepristone significantly reduces both the frequency and amplitude of LH pulses, suggesting a hypothalamic-pituitary action (Garzo et al. 1988).

In summary, mifepristone acts at multiple sites within the hypothalamic-pituitary-ovarian axis to produce the observed effects.

1.6 Endometrium and Menstruation
Endometrium is a specialised organ that is composed of two layers: a superficial functional layer and a basal layer. During the reproductive age, the superficial layer undergoes cyclical changes closely controlled by the ovarian hormones, and disappears after the menopause. It is composed of epithelial cells that line the uterine cavity, simple and tortuous glands, and stroma with cells of different lineages and vessels. Following menstrual shedding at the end of a cycle, the functional layer regenerates from the reserve cell layers in the basal zone.

Before ovulation, the oestrogen dominated proliferative phase of the endometrium is characterised by glandular and stromal mitoses. Progesterone, which is the dominant hormone in the post-ovulatory secretory endometrium, inhibits proliferation while
inducing the synthesis and the release of endometrial secretory products and neovascularisation (Critchley & Healy 1998b and references therein).

Therefore, the menstrual cycle can be defined as a repeated expression of the action of the hypothalmo-pituitary-ovarian system with associated structural and functional changes in the target tissues of the reproductive tract (Yen et al. 1991).

1.6.1 Endometrial vessels

Sex steroids have shown to have a direct effect on the vessels in the upper functionalis layer of the endometrium (Schmidt-Matthiesen et al. 1963; Johannisson et al. 1986) and these vessels may play an important role in implantation (Akerlund 1991). Following the original report by Markee in 1940, the increasing need for efficient contraceptive methods and improvement of technology related to assisted conception have renewed interest in studies of the human endometrium, including endometrial vascularisation. The rate and the volume of the blood flow to the superficial layers of the endometrium are thought to be regulated via the dynamic changes of these vessels (Ramsey 1977; Peek et al. 1992). During the pre-ovulatory period, the capillaries have a narrow lumen and are thin-walled. The discrepancy in growth rate (the five fold increase in the length of the spiral arteries while the endometrium thickness only doubling) that is apparent during the proliferative phase might be the reason for the pre-ovulatory coiling of these arteries (Markee 1940). Ovulation seems to provoke an immediate dilatation of the endometrial capillaries (Peek 1992). Herein after, in the luteal phase of the cycle, the vessels in the sub epithelial capillary plexus sustain this dilatation (up to day LH +7/ +9) up to the time of implantation (Bartelmez 1956; Sheppard et al. 1980; Seppala et al. 1991). Furthermore, this dilatation of vessels correlates with the serum progesterone levels circulating in the plasma 72 hours preceding biopsy and coincides with the development of stromal odema (Peek et al. 1992). Later, immediately preceding the onset of menses, the endothelial cells undergo contraction and degeneration (Peek et al. 1992).
1.6.2 Menstruation

The superficial or functionalis layer of the endometrium disintegrates at the end of an ‘unsuccessful’ cycle where conception has not occurred, and this process is defined as menstruation. Menses is also the climax of events that occur in the cells and vessels of the endometrium, which follow the natural withdrawal of progesterone. The current understanding of the mechanism of menstruation stems from a series of outstanding experiments performed by Markee in 1940. Using endometrial auto-transplants in the anterior chamber of the eye of rhesus monkeys, he produced a detailed account of vascular changes associated with menstrual bleeding. Subsequently two hypotheses have being advocated as the trigger mechanism for menstruation: the inflammatory hypothesis focused on pro-inflammatory cells, MMPs, chemokines and cytokines (Finn 1986; Salamonsen et al. 1999 and references therein), while the vasoconstrictor hypothesis emphasised the actions on prostaglandins (Baird 1996) and endothelins (Marsh 1996; Campbell & Cameron 1998). The vasoconstrictor hypothesis seems more plausible and thus, is progressively gaining recognition (Baird 1996, Figure 1.6; Critchley et al. 2001). Moreover, this second hypothesis integrates the previously suggested inflammatory pathway at a downstream level to the initial step, vasoconstriction.
Prostaglandins and menstruation

Prostaglandins (PGs) are a family of fatty acids synthesised from arachidonic acid (AA) which are able to elicit a wide variety of biological actions. The amount of free AA available for cells is limited; therefore, liberation of AA from precursors (present as membrane bound phospholipids) by the action of phospholipase A2 (PLA2) is one of the first and rate limiting steps in PG synthesis (Lapetina 1982; reviewed in Poyser 1992). PLA2 appears to be present in the endometrium in two different iso-forms; the calcium dependent and inducible PLA2(j) which is localised in the endometrial glands, and the calcium independent PLA2(jj) that is predominantly confined to the stroma (Bonney 1987)
dependent and inducible PLA$_2$ which is localised in the endometrial glands, and the calcium independent PLA$_2$ that is predominantly confined to the stroma (Bonney 1987a & b). Free AA is then either converted to cyclic endoperoxides by the actions of cyclooxygenase or to 5-hydroperoxy acids by the action of 5-lipoxygenase (Figure 6.1). The end products of the cyclooxygenase pathway include prostanoids (PGE$_2$, PGF$_{2\alpha}$, PGI$_2$) and thromboxane A$_2$ (Bergstrom 1968; Poyser 1992; Baird 1996). PGF$_{2\alpha}$ is a strong vasoconstrictor and a stimulant of uterine contraction. Cyclo-oxygenase is a cytoplasmic enzyme (bound to the endoplasmic reticulum) and exists as two isoforms produced by two different genes (COX-1 and COX-2) (Fletcher 1992). COX-1 is constitutively expressed in both glandular and stromal cells of the endometrium, while COX-2 expression is modulated by a variety of stimuli including growth factors and hormones and is usually limited to luminal, and glandular epithelial cells. In the endometrium, PGs are not stored, but are immediately synthesised and released, then rapidly metabolised into biologically inactive metabolites. The major site of prostaglandin synthesis during the normal menstrual cycle appears to be the endometrial glandular cell compartment (Lumsden 1984). The NAD$^+$ dependent enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH) belongs to the family of short chain dehydrogenase and is responsible for the metabolism of PGs (Ensor 1990). The endometrial release of PGs varies at different stages of the menstrual cycle, which suggests an ovarian hormonal influence.

The evidence (both in vitro and in vivo) for progesterone control of endometrial PG activity are as follows; secretory endometrium has a higher level of PGs (both PGE$_2$ and PGF$_{2\alpha}$) (Downie et al. 1974; Sing et al. 1975; Maathuis & Kelly 1978) and has a greater capacity to synthesise PGs than the proliferative endometrium (Abel & Baird 1980; Smith et al. 1981); in-vitro, progesterone suppressed both the oestriadiol-stimulated and basal PG production by endometrial explants and cells in culture (Abel & Baird 1980; Schatz et al. 1986; Kelly & Smith 1987); large amounts of PGs are released with the withdrawal of progesterone e.g. menstruation and abortion (Lumsden et al. 1983;
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Norman et al. 1991 a & b; Cheng et al. 1993 b; Poyser 1995); during pregnancy, when progesterone levels are high, basal endometrial PG production is reduced (Maathuis & Kelly 1978); antagonising progesterone in vitro shows a dose-dependent induction of PGF2α release from endometrial stromal cells (Kelly et al. 1986); the observed increase in uterine contractility after treatment with mifepristone is possibly due to increase PGs (Norman et al. 1991b; Gemzell-Danielsson et al. 1994). Therefore, under normal conditions at least, progesterone appears to enhance the PG biosynthetic capacity of the secretory endometrium, while suppressing their release. However, mifepristone still increases the uterine activity even when prostaglandin production is suppressed by indomethacin (Norman et al. 1991a).

The concentration of PG in any tissue is related to its rate of synthesis and metabolism. Thus, attempts have been made through evaluating the enzymes involved to demonstrate the mechanism by which progesterone controls endometrial PGs activity. An increase in PGDH expression (Casey et al. 1980; Kelly et al. 1994), and a decrease in the expression of COX-2 (Jones et al. 1997) is seen with the rising levels of progesterone in the secretory endometrium; progesterone has an inhibitory effect on the endometrial PLA2 activity (Bonney et al. 1987 a & b); the withdrawal of progesterone from an endometrium that has been primed with progesterone and oestradiol results in an increase in COX-2 expression (Critchley et al. 1999), while continuing exposure to progesterone is associated with low levels of COX-2 expression; following treatment with antiprogestosterone, glandular PGDH expression is markedly reduced (Cameron et al. 1996); glandular PGDH immunostaining in the endometria of women receiving mifepristone in early pregnancy also shows a reduction (Cheng et al. 1993a) while it is increased in the endometria of women using a LNG releasing intrauterine system (Critchley et al. 1998a); and finally, transfection studies have shown progesterone induced PGDH expression via interaction with a specific promotor (Greenland et al. 2000). Thus, progesterone is responsible for stimulating PGDH and suppressing COX-2 and PLA2.
Consequently, although progesterone enhances the PG biosynthetic capacity of the endometrium, withdrawal of progesterone is necessary for its full potential to be evident. Moreover, progesterone appears to exert these effects through inhibiting enzymes involved in the synthesis of PGs (PLA₂ and COX-2) and by stimulating PGDH, which metabolises PGs.

1.6.3 Mifepristone and the uterus
Mifepristone blocks the specialised uterine actions of progesterone firstly enabling the reception of the blastocyst by the endometrium and the continuous nourishment of the developing fetus and secondly containing the growing fetus until the eventual expulsion.

1.6.3.1 Effects of mifepristone on the endometrium
The endometrial effects are dependent on the dose of mifepristone used and on the time of administration in the menstrual cycle. In several studies mifepristone was tested in doses that varied between 0.5 mg and 800 mg, given either as single dose, multiple doses intermittently or continuously for several months.

Although an anti-proliferative effect has been reported in the absence of progesterone (Slayden & Brenner 1994; Brenner & Slayden 1994; Elger et al. 2000), mifepristone in general does not directly affect the endometrium. The persistent proliferative activity observed following follicular phase administration is a result of the delay in ovulation (Swahn et al. 1988). However, the endometrial effects of luteal phase (progesterone dominant) administration of mifepristone are numerous.

The endometrial effects of early luteal phase administration of antiprogesterones have been extensively investigated (Swahn et al. 1990; Gemzell-Danielsson et al. 1996; Cameron et al. 1996). Early luteal phase administration of a single or multiple doses of mifepristone disrupts endometrial maturation without affecting vaginal bleeding patterns. Treatment with early luteal phase mifepristone chronologically retards the
development of the secretory endometrium with glandular contraction, reduced luminal secretions and a decreased number of vacuolated cells (Swahn et al. 1990; Gemzell-Danielsson et al. 1994a). In the early luteal phase mifepristone inhibits progesterone induced down-regulation of PR and ER (Berthois et al. 1991; Gemzell-Danielsson et al. 1994a; Cameron et al. 1997) while antagonising the progesterone action on endometrial markers such as PGDH, which are known to be progesterone dependent (Casey et al. 1980; Greenland et al. 2000). Moreover, PGDH has been postulated as a useful marker of the “closure” of the implantation window, and the effect of mid-luteal administration on such markers may add to our current understanding of potential contraceptive actions of mifepristone. Antiprogesterone also increase AR expression in both glandular and stromal compartments (Cameron et al. 2000; Slayden et al. 2001). Furthermore, when 200 mg mifepristone is administered immediately following ovulation, uterine PGF2α release (Gemzell-Danielsson et al. 1994b), the luminal expression of COX-2 (Marions et al. 1999), and the glandular expression of leukaemia inhibitory factor (LIF: a cytokine involved in implantation process) are significantly reduced, while the immunoreactivity of Ki67 (a nuclear marker of cellular proliferation) is increased (Cameron et al. 1997). The reduction in the endometrial metabolism of oestradiol (Maentausta et al. 1993), and the reduction in the markers of endometrial maturation (Gemzelle-Danielsson et al. 1994a) suggest that early luteal phase treatment with mifepristone inhibits the normal secretory transformation of the endometrium.

In the mid-luteal phase, mifepristone at a single dose of more than 50 mg induces menstrual bleeding within 72 hours (Greene et al. 1992; Schaison et al. 1985; Shoupe et al. 1987). Luteolysis was incomplete in two thirds of the subjects, and they experienced a further episode of vaginal bleeding at the expected time of menses. In the remainder there was complete luteolysis with only one episode of bleeding. Thus the vaginal bleeding observed after mifepristone without a persistent decrease in the circulating progesterone values, appears to be due to a direct effect on the endometrium. Observations similar to those seen before menstruation such as shrinkage of glandular lumen, leucocyte infiltration and vascular necrosis have been reported (Li et al. 1988;
Swahn et al. 1988). Johannisson et al. in 1988 suggested that the menstrual bleeding induced by mifepristone in mid-luteal phase is a direct endometrial vascular effect. Following treatment with 50 mg of mifepristone in the mid-luteal phase (days 20 – 23), they described a significant reduction in the capillary luminal area and diameter associated with degenerative changes in the endothelial cells, which preceded the menstrual shedding. They interpreted the decreased capillary luminal area as vasoconstriction. These changes did not always accompany regressive changes in the adjacent stroma. This effect of mifepristone on the endometrium (at a time when the endometrial progesterone receptor level is relatively low) is poorly understood, and has hardly been investigated. Mifepristone may induce bleeding by increasing local concentration of prostaglandins, which are potent vasoconstrictors. Indeed, the evidence is available for similar action of mifepristone on local PG in the early pregnancy where it has been known to reduce the PG metabolising enzyme PGDH (Cheng et al. 1993a). Moreover, in the early-luteal phase progesterone values are relatively low, and as a consequence the PR and ER expression is maximal, while the converse is true for the mid-luteal phase of the cycle. Therefore, in the early luteal phase mifepristone may prevent the effects that are yet to be exerted by progesterone, whereas in the mid-luteal phase it may antagonise the actions of progesterone, which at that time appears to be the suppression of release of PGs.

When given as a daily pill, the threshold dose of mifepristone required to disturb the endometrial development appears to be 1 mg/day (Batista et al. 1992b; Croxatto et al. 1993). 5 mg weekly dose will also be sufficient to inhibit the normal secretory development of the endometrium (Gemzelle-Danielsson et al. 1996).

In the absence of progesterone, mifepristone induced agonist-like secretory changes in the oestrogen primed postmenopausal endometrium suggests it to be a partial rather than a pure antagonist (Gravanis et al. 1985).
1.7 Contraceptive potential of mifepristone

Progesterone is essential for the establishment and the maintenance of pregnancy, thus the anti-progesterone mifepristone has multiple potential anti-fertility actions. Mifepristone is potentially contraceptive by virtue of its effects either on the hypothalamic-pituitary-ovarian axis, and/or, by direct effects on the uterus. The hypothalamic-pituitary effects are particularly apparent in the follicular phase of the cycle, where mifepristone in large doses inhibits gonadotrophin secretion, consequently arrests follicular-growth and prevents or delays ovulation (Shoupe et al. 1987; Permezel et al. 1989; Stuenkel et al. 1990). The endometrial effects are largely confined to the luteal phase. Mifepristone is able to retard the normal endometrial development or to induce endometrial shedding depending on the time of administration in the luteal phase. Moreover, the recently reported actions of mifepristone on the embryo, sperm and tubal function, may also have additional contraceptive effects (Yang et al. 1994; Baldi et al. 2000; Spitz 2000).

The use of mifepristone in the following contraceptive options has been explored.

1.7.1 Occasional menstrual regulation

Some have suggested using mifepristone early in the pregnancy when menstruation is delayed (up to 6 weeks of gestation) as a method of contraception. Women, even after having sexual intercourse during the fertile period of the cycle do not fall pregnant each month. Therefore, a menstrual regulatory agent that has to be taken occasionally (only if the conception had occurred and menstruation is delayed) can be attractive to some. However, this approach of using mifepristone alone to interrupt a pregnancy in the first 6 weeks was neither very effective (success rates about 85%) nor acceptable to many women (Couzinet et al. 1990; Spitz et al. 1993a; Glasier et al. 1998). Some reports have suggested that if given in combination with a prostaglandin to women with menstrual delay of up to 11 days, mifepristone may have a role as an occasional ‘menstrual regulator’ (WHO 1995), while others have shown that this regimen is ineffective when
given at the expected time of menses (Dubois et al. 1988; Swahn et al. 1999). Although this method has an important practical implication in those countries that allow menstrual regulation even when abortion is prohibited, its legality or acceptability in general is likely to be limited.

1.7.2 “Once-a-month” luteal phase administration
Mifepristone may prevent implantation in luteal phase either by preventing the development of the normal secretory endometrium, or by inducing endometrial shedding (mid luteal phase). When administered immediately following ovulation, although able to alter the endometrial development causing a delay in the emergence of the implantation window, a single-dose of mifepristone 200 mg did not affect the menstrual cycle length (Swahn et al. 1990). Preliminary results of clinical trials have confirmed the contraceptive efficacy of this approach. In the first study, after taking 200 mg of mifepristone on day LH+2 only one pregnancy occurred in 124 cycles, in which women were exposed to the risk of pregnancy (Gemzelle-Danielsson et al. 1993). Although many women find this approach to be an attractive option, the feasibility of administering mifepristone in this manner is deterred by the requirement of precise detection of the LH surge. An easy, highly effective, and cheap method to define the LH surge (probably in a urine sample) would be necessary for this approach to be suitable for wide-spread use.

1.7.3 “End of the month” regular late luteal phase administration
Although a simpler, and more practical approach to the above, since it will induce menstrual bleeding at the expected time of the month (Croxatto et al. 1987), the existing data on the contraceptive efficacy of mifepristone as a regular ‘end of the month pill’ in the late luteal phase are disappointing. Reported failure rates of approximately 5% per treatment cycle and 17% - 19% per pregnancy cycle (Ulmann et al. 1987; Dubois et al. 1988; Couzinet et al. 1990; Reviewed in Swahn et al. 1996b), are comparable to the failure rates of mifepristone when used on its own to terminate early pregnancy.
Addition of 400 µg misoprostol to 200 mg mifepristone used regularly at the expected time of menses did not improve the success rates (Swahn et al. 1999).

1.7.4 Emergency contraception
In theory mifepristone approximates the ‘ideal’ emergency contraceptive. If unprotected intercourse occurs before ovulation it is able to prevent pregnancy by delaying ovulation. Conversely, if unprotected intercourse occurs after ovulation, mifepristone may prevent implantation via its endometrial action. Two randomised trials comparing 600 mg of mifepristone with the standard Yuzpe regimen of combine oestradiol and Levonorgestrel have confirmed its efficacy as an emergency contraceptive, when given within 72 hours of unprotected intercourse (Webb et al. 1992; Glasier et al. 1992). They also reported a significantly low incidence of side-effects with mifepristone. However, the main drawback with this dose is the delay in the onset of subsequent menses, presumably when it was administered in the follicular phase. Reassuringly, a recent large multi-centre study comparing 10, 50, or 100 mg doses of mifepristone reported that the delay in the onset of the next menses and the side effects are dose-related, and the reduction of the dose to 10 mg did not compromise the efficacy (WHO 1999).

1.7.5 “Once-a-week” use
Weekly doses of either 2.5 mg or 5 mg mifepristone can delay endometrial maturation and the appearance of progesterone dependent markers of the endometrium, without affecting ovulation, hormonal profile or the menstrual cycle (Gemzell-Danielsson et al. 1996). However, in unprotected women the regimen of 5 mg every week failed to demonstrate efficacy in preventing pregnancy (Marions et al. 1998). When the weekly dose is increased to 10, 25 or 50 mg a considerable disruption of the menstrual pattern was observed with inconsistent inhibitory effect on ovulation (Spitz et al. 1993b & 2000; Chen et al. 1997). In view of this data, intermittent administration of mifepristone has been abandoned as a potential contraceptive method.
1.7.6  Daily low dose and cyclical administration

Continuous daily doses of above 2 mg of mifepristone reproducibly suppresses ovulation (Ledger et al. 1992; Corxatto et al. 1993) and, clinical trials have confirmed its contraceptive effect (Brown et al. 2002). A 5 mg dose consistently inhibits ovulation, while ovulation was permitted in 10% of the cycles with 2 mg dose. However, probably due to the additional anti-fertility effect on the endometrium, both doses are effective as contraceptives (Ledger 1992; Corxatto et al. 1993). When women either took 2 or 5 mg mifepristone daily as their only method of contraception, there were no pregnancies in the 200 months of treatment (Brown et al. 2002).

Cyclical daily regimens of mifepristone in doses of 5 or 10 mg for the first 15 days of the cycle followed by a progestin for the subsequent 14 days had been tested with the view to produce a more ‘natural’ regular bleeding pattern. Although this regimen resulted in a regular bleeding pattern as expected in a significant number of patients, it did not suppress ovulation (Croxatto et al. 1996 & 1998). Furthermore, the contraceptive efficacy of this approach remains to be investigated.

1.7.7  Male contraception

Mifepristone opposes the effects of progesterone on calcium influx, sperm motility and the acrosome reaction by inducing a rapid, transient, and dose dependent decrease of intracellular free calcium and a drop in the fraction of hyperactivated sperm (Yang et al. 1994). This approach however, has not been pursued because of difficulty in reproducing these results.

1.7.8  Other clinical uses of mifepristone

Mifepristone has been widely used for pregnancy termination in the first and second trimester and it is licensed for this use in many countries in the world. The other possible uses of mifepristone include induction of labour, cervical ripening prior to suction termination of pregnancy or surgical intervention of the uterine cavity such as
hysteroscopy, for the treatment of endometriosis, fibroids, and progesterone receptor positive breast cancer. Potential non-gynaecological uses of mifepristone include reduction of intra-ocular pressure in glaucoma and steroid induced myopathy, treatment for PR positive brain tumours such as meningioma, and Cushing’s disease due to ectopic ACTH secretion or adrenal carcinoma. These effects reported are believed to be secondary to its anti-glucocorticoid properties (Sakar 2002).

1.8 Emergency Contraception

Emergency contraception is defined as the use of a medication or a device to prevent pregnancy once sexual intercourse had occurred (Glasier & Baird 1997) yet many perceive EC as abortifacients. This is because the action is taken after, rather than before intercourse. However, the definition of an abortion is interference with a pregnancy after implantation and, the time from ovulation to implantation takes an estimated seven days. Since the ovum, once ovulated, is capable of being fertilised for only up to 24 hours, use of an EC within 72 hours of sexual intercourse (that is responsible for fertilisation) cannot induce an abortion. Nevertheless, this confusion and misinterpretation among the providers and the users of EC may be the main impediment to the widespread use of emergency contraceptives, which could prevent millions of unwanted pregnancies in the world (Trussell et al. 1992). A detailed understanding of the precise mechanism by which an emergency contraceptive prevents a pregnancy can be advantageous in clarifying these misconceptions.

Unfortunately, the exact mechanism of action of most widely used emergency contraceptives is still poorly understood.

1.8.1 Methods available

Not using a contraceptive at all or failures in a method used (e.g.: burst condom) are common reasons for a woman to seek emergency contraceptives. Today, the available methods of post-coital contraception in the UK include Yuzpe regimen of a combination of oestrogen (ethinyl oestradiol) and progestin (Levonorgestrel), progestin alone
(Levonorgestrel) and the emergency insertion of intrauterine device (IUD). Although the methods involving steroid hormones eclipse the highly effective yet inconvenient IUD in post coital contraception, they are associated with high incidence of side effects. The data available on the safety of these regimens when used as EC are limited, yet at least theoretically they appear to be extremely safe. All the above regimens can be used as long-term contraception and there is an abundance of data indicating their safety when used in these ways (reviewed by Turner & Ellerston 2002). Furthermore, WHO and the US Food and Drug Administration, have declared that EC has an excellent safety profile in nearly all women. In their review of EC safety, Turner et al. went on to suggest that whether carried to term or terminated, an unwanted pregnancy is more hazardous to the well-being of the woman than the risks posed by taking EC.

1.8.2 Efficacy of EC

Generally efficacy of EC is defined as how well the method prevents pregnancies in ideal conditions. There are no placebo-controlled trials quantifying the efficacy of emergency contraceptives. Conventionally, the effectiveness of emergency contraceptive methods have been calculated by comparing the observed number of pregnancies with the number expected in the absence of treatment (Yuzpe & Lancee 1977; Ho et al. 1993; Glasier et al. 1997; WHO 1998). In most studies to date, the anticipated number of pregnancies is estimated by using external data based on two studies of couples planning pregnancy (Barrett & Marshall 1969; Wilcox et al.1988). The two studies calculated the probability of pregnancy resulting from unprotected intercourse on a particular day of the cycle, relative to the predicted day of ovulation. This approach poses some obvious problems. Only women with regular cycles can be used to estimate the efficacy; the validity of the results is directly dependent on the accuracy of the reported menstrual data; the estimates are sensitive to errors in identifying the day of ovulation (Dunson et al. 1999); other factors can affect the probability of pregnancy (e.g. was there an actual spillage of semen, fertility of the couple) that are unrelated to the timing of intercourse (Schwartz 1980) and conception is used synonymously with implantation, not
fertilisation (Trussell et al. 1998). It has been suggested that the true effectiveness of EC may be higher than the calculated figure for the following reasons; the estimated number of pregnancies tend to be smaller since the estimates are based on studies which are deficient; the number of pregnancies observed tend to be higher due to women either being pregnant at the time EC was administered or they become pregnant by a subsequent act of intercourse (Trussell et al. 1998). Conversely, the argument remains that the effectiveness has been overestimated because the risk of pregnancy without EC is less than that calculated from Dixon / Wilcox tables. The women in Dixon / Wilcox estimates were trying to get pregnant, without any history of infertility and had regular menstrual cycles. Many women who present for EC are not at risk because they are nowhere near ovulation, and they may not have had vaginal intercourse with sperm in the vagina. Thus the effectiveness of EC may be overestimated.

Table 1.1 illustrates the reported efficacy of the different methods.

1.8.3 Mechanism of action of emergency contraceptives

An emergency contraceptive can be effective by inhibiting or disrupting ovulation / fertilisation, interfering with the transport of the blastocyst, or inhibiting its implantation in the endometrium. Sex steroids have several effects on the ovulatory cycle and on the endometrium depending on the time of administration, hence have been used as emergency contraceptives.

Today, a quarter of a century since its initial discovery, the mechanism by which Yuzpe regimen (Yuzpe & Lancee 1977) acts as an EC remains unclear. Although different theories are on offer, based on reported effects after administering the Yuzpe regimen such as, interference with ovulation (Ling et al. 1983a; Rowlands et al. 1986; Swahn et al. 1996a; Croxatto et al. 2002), or disrupting endometrial maturation (Ling et al. 1983b), they appear to be insufficient to fully explain the observed clinical efficacy of the regimen (Trussell & Raymond 1999). The minor changes observed in the endometrium
<table>
<thead>
<tr>
<th>Regimen</th>
<th>Time after intercourse</th>
<th>Reported efficacy</th>
<th>Source of data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yuzpe</td>
<td>Within 72 hours</td>
<td>72.5-74.1% of expected no of pregnancies are prevented</td>
<td>Meta-analysis of 7 and 10 studies (Trussell 1998; Trussell 1999b)</td>
</tr>
<tr>
<td>LNG 0.75 mg taken twice</td>
<td>Within 72 hours</td>
<td>Similar to Yuzpe</td>
<td>2 randomised trials involving a total of 1386 women (Ho 1993; WHO 1998)</td>
</tr>
<tr>
<td>12 hours apart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mifepristone</td>
<td>Up to 120 hours</td>
<td>85% to 100%</td>
<td>3 randomised trials involving a total of 2281 women (Glasier 1992; Webb 1992; WHO 1999)</td>
</tr>
<tr>
<td>Single dose of 10 – 600 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danazol 400 - 800mg x2 or</td>
<td>Within 72 hours</td>
<td>failure rates of &lt;1% to 100%</td>
<td>2 randomised trials, Webb 1992 (n= 193) shows no effect. Zuliani 1990 (n &gt;1700) shows &lt;1% failure rate</td>
</tr>
<tr>
<td>400mg x 3 taken 12 hours apart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper intra uterine device</td>
<td>Up to 5 days after the earliest estimated day of ovulation</td>
<td>Failure rate &lt;1%</td>
<td>Meta – analysis of 20 published studies involving more than 8000 women (Trussell 1995)</td>
</tr>
<tr>
<td>High dose Oestrogen</td>
<td>Within 72 hours</td>
<td>Similar to Yuzpe</td>
<td>Randomised trial involving 250 women (Dixon 1980)</td>
</tr>
</tbody>
</table>
during the implantation period following Yuzpe (changes in endometrial ER and PR (Kubba et al. 1986), MUC-1 expression (Raymond et al. 2000), minor morphological changes (Raymond 2000)) are inconsistent (e.g. Swahn et al. 1996b; Marion et al. 2002) and may even be incapable of preventing implantation.

1.8.4 LNG as an emergency contraceptive

Two consecutive doses of Levonorgestrel (LNG) 0.75 mg administered 12 hours apart within 72 hours of the unprotected coitus, has been shown to be an effective method of post coital contraception (PCC). This regimen acquired a licence as an emergency contraceptive in the UK during the latter part of the year 2000. It is also being used for this purpose in Eastern Europe, the Far East and many developing countries. There have been only 2 trials (Ho et al. 1993; WHO 1998) published to date comparing the efficacy of this regimen with the standard method of emergency contraception, the Yuzpe regimen. The two studies showed that LNG performed effectively as an emergency contraceptive and had fewer side effects than the Yuzpe regimen. Neither of the two studies was designed to identify the possible mechanism(s) of action by which LNG exerts its post-coital contraceptive effect. Hence, the mechanism of action of LNG used in this manner remains unknown. Furthermore, in some countries, introduction of EC requires evidence on mechanism of action that suggest further exploration into the effect of LNG would be advantageous.

1.8.5 Possible EC mechanisms of action of LNG

1.8.5.1 Effect of LNG on the LH surge, ovulation & luteal function

In physiological doses progesterone initially promotes the oestrogen elicited mid-cycle LH surge in oestrogen treated ovariectamised women (Odelle & Swerloff 1968), and in women with intact ovaries (Permezel et al. 1988). Long-term administration however, enhances the negative feedback effects of E2 (Dierschke et al. 1973; Clifton et al. 1975; March et al. 1981). In combination with oestrogen, continuous administration of progestin (in the form of COCP) abolishes the pre-ovulatory LH surge and suppresses
the LH levels down to those observed during the luteal phase (Swerdloff et al. 1968; Klopper et al. 1973). The Yuzpe regimen also has been shown to suppress or delay the LH surge and ovulation (Croxatto et al. 2002). On their own, synthetic progestins (Norgestrel) at supra-physiological doses have been shown to abolish the mid cycle LH surge leading to anovulation and delay of the onset of the subsequent menses (Weiner et al. 1975). The lowest plasma level reported to inhibit ovulation appears to be 1 nmol/L (Nilsson et al. 1980). Repeated supra-physiological doses of (0.75 mg) LNG in the peri-ovulatory phase of the cycle delayed the LH surge and disrupted ovulation and normal luteal function in some women (Landgren et al. 1989). Others have also reported a decrease in the mid-luteal LH surge after treatment with LNG in the follicular phase, with the exception of some women this was associated with a normal luteal phase pregnanediol excretion (Kessaru et al. 1974; Sopna et al. 1975; Marions et al. 2002). Progestins suppressed FSH induced E2 production by rat granulosa cells in culture (Schreiber 1980; Fortune 1983), decrease synthesis of oestrogen pre-cursor (androstendione) and inhibit follicular development in monkeys, despite FSH enhancement (Goodman et al. 1982; Weibe et al. 1984). These observations suggest that the pre-ovulatory administration of LNG may interfere with the E2 signal that precedes the LH surge.

During the luteal phase of the cycle, progestins depress basal LH secretion (Weiner et al. 1975). In the human, basal level of Lutenizing Hormone (LH) is essential for the normal secretory function of the corpus luteum (Vande Weile et al. 1970). In 1971 Johansson et al., suggested that synthetic progestins may have a possible post ovulatory contraceptive effect by interfering with normal luteal function and decreasing plasma progesterone. Many have reported a shorter luteal phase after progestin administration, which accompanied a lower than usual luteal progesterone production (Johansson 1971; Kessaru et al. 1975; Durand et al. 2001).

Table 1.2 illustrate the effects of synthetic progestins on the menstrual cycle reported in the literature.
Table 1.2  Effects of synthetic progestogens on the menstrual cycle

<table>
<thead>
<tr>
<th>Reference (n = subjects)</th>
<th>Progestin / Dose</th>
<th>The cycle day (cd) of administration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weiner et al. 1980 (n = 4)</td>
<td>d-norgestrel 40 mg implant</td>
<td>Day 5 onwards 90 + days</td>
<td>Ovulation suppressed LH peak suppressed</td>
</tr>
<tr>
<td>Landgren et al. 1990 (n = 20)</td>
<td>Norethisterone 300 mcg (n = 10) LNG 30 mcg (n = 10)</td>
<td>Day 7 - 10</td>
<td>NET; In 4 both LH peak and pregnanediol suppressed. Follicular phase was prolonged in one; area under the curve (AUC) increased for E2, decreased for Pregnanediol (P₄); Endometrial sub-nuclear vacuolation and decreased gland mitosis LNG; No LH peak in 3 and pregnanediol suppressed in 4. One woman had a prolonged follicular phase. Decreased AUC of P₄ in all No endometrial effects</td>
</tr>
<tr>
<td>Landgren et al. 1989 (n = 72)</td>
<td>0.75 mg LNG</td>
<td>1. n = 17 2. n = 17 3. n = 18 4. n = 19</td>
<td>1. 17/17 normal cycle 2. 3/17 anovulatory, 7/17 incomplete luteal activity, 7/17 normal 3. 5/18 anovulatory, 6/18 incomplete luteal activity, 7/18 normal post-treatment cycle 1/18 anovulatory, 1/18 incomplete luteal activity 4. 19/19 normal cycle</td>
</tr>
<tr>
<td>Johansson et al. 1971 (n = 7)</td>
<td>Norethisterone Norgestrel MPA Chlormadinone</td>
<td>All post-ovulation LH+3 onwards</td>
<td>Shortened luteal phase Reduced total P₄</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>Kesseru et al. 1974 (n = 11)</td>
<td>d-norgestrel 400 mcg</td>
<td>Group A</td>
<td>Group A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. 10 (n = 4)</td>
<td>1. 1/4 no LH peak } All had</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. 6 &amp; 10 (n = 4)</td>
<td>2. 3/4 no LH peak } normal luteal P₄ levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. 6,10,14 (n = 1)</td>
<td>3. &amp; 4. Both decreased LH peak</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. 6,10,14, 18 (n=2)</td>
<td></td>
</tr>
<tr>
<td>Group B (n = 9)</td>
<td>PCC tablets 5 – 12 per subject</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 showed no LH peak, significantly low P₄ levels if &gt;5 tablets were taken.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra-cervical sperm penetration effect seen 9 – 10 hours (h) after ingestion.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical mucus changes starting at 3-4h and max change at 9h post-ingestion.</td>
<td></td>
</tr>
<tr>
<td>Craft et al. 1975 (n = 8)</td>
<td>Norgestrel 750 mcg</td>
<td>Follicular phase up to 9 doses</td>
<td>7 had delayed menses, and showed no LH peak or delayed LH peak. All 9 women also reported irregular vaginal spotting. One had short luteal phase.</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment</td>
<td>Phase/Outcome</td>
<td>Summary</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Spona et al. 1975</td>
<td>d-norgestrel 400 mcg</td>
<td>1. 12&lt;br&gt;2. 10 &amp; 12&lt;br&gt;3. 10, 12 &amp; 14&lt;br&gt;4. post ovulatory</td>
<td>1. No LH peak but normal rise in $P_4$&lt;br&gt;2. Abnormal LH rise and short luteal phase&lt;br&gt;3. No LH peak, delayed rise in $P_4$ (may suggest delayed ovulation)&lt;br&gt;4. No effect</td>
</tr>
<tr>
<td>Durand et al. 2001</td>
<td>LNG (EC regimen)</td>
<td>Group A (n = 15, d 10)&lt;br&gt;Group B &amp; C (n = 11 during LH peak)&lt;br&gt;Group D (n = 8, late follicular phase)</td>
<td>A = 12 anovulatory, remaining 3 short luteal phase and low luteal $P_4$.&lt;br&gt;B and C = no effect&lt;br&gt;D = low luteal $P_4$, no effect on LH peak</td>
</tr>
</tbody>
</table>
| Marions et al. 2002| LNG (EC regimen)    | Administered on LH-2                                                          | Low LH peak, normal luteal $P_4$
One short luteal phase. |
1.8.5.2  **Effect of LNG on endometrium**

The main effects of short-term administration of LNG on the endometrium seem to be secondary to the delaying of ovulation. Landgren and colleagues (Landgren et al. 1989) showed that follicular phase administration of multiple doses of 0.75 mg LNG suppresses the secretory activity of the endometrium. However, the same dose of LNG did not affect the endometrium when given in the luteal phase. Glandular atrophy, stromal decidualisation and epithelial cell inactivation have been observed after chronic local exposure of the endometrium to LNG (Pakarinen et al. 1995). Locally administered LNG also induces expression of the potent contraceptive glycoprotein Glycodelin A during the implantation period (Mandelin et al. 1997). At high doses (20 – 40 ng/ml) progesterone inhibits the growth of human capillary endothelial cells in culture (Peek et al. 1995). Similarly, although LNG stimulates angiogenesis in the dose range 100 pmol/l to 10 nmol/l, this effect was reversed at higher doses (Hague et al. 2002). Recently, Marions et al. reported that the emergency contraceptive dose of LNG administered in the peri-ovulatory period did not change any of the endometrial parameters including integrin α4 and β3 (these are surface receptors for extracellular cell adhesion proteins to cytoskeletal components and are involved in blastocyst implantation), COX 1, COX 2, progesterone receptors, pinopodes and Dolicos biflorus agglutinin lectin binding (illustrates the secretory activity of the endometrial cells) (Marions et al. 2002). Therefore, the question remains as to whether any alterations occur in the endometrium after taking the emergency contraceptive regimen of LNG at other times in the cycle, and whether those changes are sufficient to prevent implantation and contribute to the observed contraceptive efficacy of LNG used in this manner.

1.8.5.3  **Effect of LNG on cervical mucus**

The endocervical mucosa of the human uterine cervix consists of pockets of columnar epithelium (comprises different types of secretory cells) forming the so called ‘crypts’ that vary in structure with age, disease and stage of the menstrual cycle. The secretions from these cells (principle component of the cervical mucus) are regulated by the
circulating ovarian hormones, thus show a cyclical variation. Oestrogens stimulate secretion of acellular, elastic, watery mucus, and progesterone blocks the oestrogen-induced secretory activity of the cervical mucosa to produce a cellular, viscous, scanty mucus that is hostile to sperm. It has been proposed that cervical mucus plays several key functions including: aiding sperm penetration at or near ovulation while interference with entry at other times; protecting sperm from the hostile vaginal environment; supplying nutrients; selecting sperms (by working as a filter) on the basis of differential motility; storing sperm; and initiating sperm capacitation (WHO laboratory manual 1992). The effect of progestagens on cervical mucus and on the cervix is well documented and this is thought to be the main mechanism by which the progestagen only pill exerts its anti-fertility action (Odeblad et al. 1972; Daunter et al. 1976; Moghissi et al. 1973). However, to be of an emergency contraceptive significance, these effects require treatment to be initiated relatively early once intercourse has taken place. The transportation of sperm from the vagina to the fallopian tube seems to be extremely rapid (Kuntz et al. 1996), so even if LNG has an effect on cervical mucus that interferes with sperm penetration, that action is unlikely to prevent a pregnancy when taken some 12 - 72 hours after coitus.

### 1.8.5.4 Effects of LNG on the sperm

Although it is widely accepted that progesterone has an important role in sperm function, the precise mechanism by which it exerts this action is not known. In order to fertilise the ovum, the spermatozoa is required to penetrate through the cumulus mass and progesterone is a main secretory product of the cumulus matrix. Progesterone stimulates a rapid influx of calcium into the spermatozoon via a non-genomic action (Plant et al. 1995; Tesarik et al. 1996; Aitkin et al. 1996), induces an acrosomal reaction (Oehninger et al. 1994), and increases hyperactive motility of the sperm (Uhler et al. 1992). An impaired response of the spermatozoon to progesterone is a clinically well-recognised defect in the infertile male (Tesarik et al. 1992; Falsetti et al. 1993). Given their total lack of transcriptional ability, it would appear that in the sperm the non-genomic
progesterone receptor would have to be responsible if LNG were to have an emergency contraceptive effect.

1.9 Patient Compliance

1.9.1 Definitions and history

Generally, compliance is defined as yielding; complaisance; or submission (Chambers dictionary). In a clinical context, it is described as following the instructions of the health care professional (Haynes 1979) and is synonymous with adherence; maintenance; fidelity and concordance (Sackett 1976). Most of these definitions denote obedience – “following doctors orders”. Therefore, the word “compliance” can be associated with somewhat ‘negative’ connotations for the patient. If the patient perceives the very concept of compliance as one that is morally and psychologically flawed, non-compliant behaviour can be as deviant as compliance in itself (Mullen, BMJ editorial 1997). However, to the prescriber, these definitions explain the patient’s behaviour and, also describe the relation between prescription and the clinical outcome (Eisen 1991).

Patient adjusting or discontinuing the therapeutic regimen without professional medical advice is not a novel concept. Rather, it dates back to the period before Hippocrates (Wright 1993). Therefore, human nature may be at blemish for individuals selecting to follow the many deviations in the prescribed therapeutic plan. Whatever the reason is, non-compliance has serious implications for drug regulation, research and medical care.

1.9.2 The magnitude of the problem of non-compliance

Medicines are rarely taken as prescribed (Marinker 1997). This may adversely affect the quality of medical care and may waste resources. Over 50% of the patients suffering from chronic disease do not take the prescribed medicines in doses sufficient to achieve the therapeutic effect (Sackett 1979). Reflecting on their own adherence behaviour, a group of health care professionals who formed a working party on medicine taking admitted that their behaviour rarely coincided with the prescribed medical advice
Introduction and literature review

In any medical discipline, non-compliance disrupts and mitigates the benefits of the preventative or curative regimen offered. Since the medical profession is reportedly poor in identifying non-compliers (Gilbert et al. 1980; Bonner & Carr 2002), consequences of non-compliance can lead to unnecessary diagnostic tests or additional treatment procedures, thus generating further financial wastage. In 1995 Rosenberg and colleagues estimated $2.6 billion as the cost associated with unintended pregnancies that occurred due to poor compliance with the oral contraceptive pill.

Non-adherence to a protocol can have an undesirable influence on study power and interpretation of results. When undetected, poor compliance can invalidate results of efficacy studies (Rudd 1991; Urquhart 1991; Dominik et al. 1999).

Although many investigators have shown an active interest in the field of patient compliance since mid 1960's, the ambiguity and the divergence in their viewpoints have only confused our current understanding of the nature and the extent of the problem. Compliance has been described in terms of problems of omission (e.g.; failure to report an event, failure to take a medicine or to report an adverse effect) or commission (e.g.; inaccurately over reported events, overdose) (Green et al. 1991). It can be linked to the clinical outcome as either the cause (e.g.; a reason for a medicine that is effective for a certain population being labelled as ineffective) or the effect (e.g.; side effects of a treatment causing patients to be non-compliant with the regimen) (Hurley 1991). Patient and behavioural parameters (age, life style (Potter 1991; Rapoff & Barnard 1986; Meichenbaum 1987)), regimen parameters (dose interval, route of administration (Puller et al. 1988; Rapoff 1989; Cromer et al. 1989)) and response feedback parameters (side effects, positive drug effects) are recognised by some as the determinants of compliance (Haynes 1979; Hurley 1991).

Unfortunately, this renewed interest has not delivered the desired new concepts for improving patient compliance, yet it seems unfair to criticise the studies that explored
the issue of compliance for their poor methodology. There is a lack of sound techniques to quantify compliance. The existing knowledge of quantitative and qualitative features of non-compliance behaviour that is prerequisite for a 'good' study design has been limited. Thus future research should be directed towards rectifying these deficiencies. Otherwise, it is difficult to conjecture progress.

1.9.3 Measuring and monitoring compliance
There are direct and indirect methods of quantifying compliance yet limitations exist in all these methods.

1.9.3.1 Direct methods
Direct observation of the patient ingesting a medicine, biologic parameter affected by the medication and physiologic changes induced by the medication, therapeutic drug monitoring using blood or urine tests, unannounced spot checks on patients, and clinic attendance are used in various instances as methods to measure compliance directly (Spilker 1991).

Direct observations by the investigator is only suitable for inpatient studies and a few studies where the patients are required to attend the clinic to obtain infrequent doses of medicines. In our study patients took the 200 mg of mifepristone tablet under the direct supervision of the investigator. Even under these exceptional conditions, some patients can still be non-compliant (e.g.; pretend to put the medicine in the mouth but spitting it out later) (Spilker 1991). Furthermore, methods such as measuring biological markers and drug levels in biological fluids cannot disclose the chronic adherence behaviour and several other variables apart from compliance may affect pharmacokinetic values (Cramer 1990). Spot checks in particular are a potential invasion of patient’s privacy.
1.9.3.2  **Indirect methods**

Patient self-report (SR), pill counts (PC), the frequency of obtaining repeated prescriptions and a variety of electronic devices (For example; devices that monitor self testing of daily urine or record removal of each tablet) have been used to ascertain compliance indirectly (Pullar 1988; Waterhouse et al. 1993; Simmons et al. 2000). Although SR remains the most widely used tool measuring compliance in clinical practice and in research, many have reported significant overestimation of compliant behaviour with SR (Potter et al. 1996; Waterhouse et al. 1993; Simmons et al. 2000). A group of OC users self reported missing only 34% of the pills, when the data from an electronic device with diary entries showed 75% of the pills being missed (Potter 1996). Similarly, when compared to a micro-electronic monitoring system, traditional measures (SR and pill counts) significantly over estimated the adherence behaviour in a group of women receiving oral tamoxifen for treatment of invasive breast cancer (Waterhouse et al. 1993).

Anyhow, finding a method that measures compliance efficiently and that can be applied universally is just the beginning. One has to then, find a reference point in compliance, to discern compliers from non-compliers.

1.9.4  **Non-compliance in research studies**

Poor patient compliance is one of the most commonly overlooked problems in clinical research (Dahlstrom & Eckernas 1991). If undetected, it can make the results of a clinical trial invalid. Furthermore, the results from a compliant patient population may not be appropriate to extrapolate to a non-compliant group and vice versa (Dominik et al. 1999). The significance of the occurrence of non-compliance with a particular aspect of a trial depends on its relationship to the outcome. Though it is irrefutably wrong to overlook the issue of non-compliance in our approach to patient care, due to the complex nature of behaviours that involves, many doctors and investigators choose to ignore it completely.
Randomised controlled trials may not necessarily recreate or mirror clinical practice in general. They are highly artificial scientific endeavours hence; their interpretation is dependent on the possibility of the scientific principle being applied in the clinical setting (Hurley 1991). However, common sense suggests that the prevalence of non-compliant behaviour is likely to rise in the general population compared with that of a typical research study.

For a volunteer, taking part in a research study usually involves extra effort. Volunteers in clinical trials are required to attend extra clinics, answer questions, collect samples such as daily urine, allow invasive tests such as blood tests, and keep accurate records of daily events in order to comply with the study protocol. People volunteer for research studies for various reasons. Some may seek positive health benefits from a 'new' treatment in a study, and this may become their motivation to volunteer. However, in most randomised trials with a control arm and in preliminary biomedical studies the participants are aware that they may not experience a favourable effect from the treatment. They may expect other benefits; such as health screening, individual attention from health care professionals, first-hand knowledge into new discoveries, and financial gains; or they may just be altruistic. Anyhow, if the volunteer perceives the sacrifices necessitated by being involved in a research study to be more burdensome than their perception of the personal or other benefits of the study, non-compliance will be inevitable.

Even though non-compliers are more likely to withdraw or drop out from a study, they should not be excluded from the analysis of phase III trial results. The dropout rate may indirectly provide extra information on the true effect of a treatment regimen that can be expected in general use. In phase I trials however, non-compliance can affect the findings thus non-compliers may be withdrawn.
1.9.5 Compliance with contraceptives

Naturally, an improved understanding of the causes of non-compliance can aid in finding means to achieve better compliance. In order to introduce a new contraceptive method successfully, effort must be made to minimise “adverse events” and particularly side effects, in order to maintain a good level of compliance. What factors will interest women in a new method of contraception and encourage them to continue using it? Conversely, what will confound its success and dissuade women from using it? Inferences can be drawn from the information on acceptability of existing contraceptive methods among different groups. We can assume that life-stage, age, level of education and personal experience might play an important role in women’s knowledge of and attitudes towards contraception. Moreover, the reliability, accessibility, and minimal interference with sex life may be major motives for the choice of contraceptive.

The psychosocial literature on health behaviour concludes that the “health belief model” is the best explanation for non-compliant behaviour undertaken by a healthy person (Kasl & Cobb 1966). If this model is applied to the contraceptive compliant behaviour, following key elements have to be recognised.

1. The users perception of their own susceptibility (probability) of pregnancy.
2. Their attitude towards pregnancy and the perception of severity of the consequences of pregnancy (physical, financial, psychological and social).
3. Their personal definition of compliance and understanding of the importance of perfect compliance.
4. Their perception of the effectiveness of the contraceptive method if properly used.

The users of contraceptive regimens are usually young and healthy and, for the individual, the serious outcome of non-compliance (pregnancy) is rare. They have to adhere to a contraceptive regimen on a long-term basis. They may find the daily requirement for medication taking as in oral contraceptive pills or daily urine testing as in Personal Hormone Monitoring device to be complicated or inconvenient. Side effects of the method may not be acceptable and, they may not have faith in the effectiveness of
the method. Considering these odds, it is not difficult to infer why compliance problems are frequent.

Most of the literature on contraceptive compliance is concentrated on the combined oral contraceptives (COC). For example, Emans and colleagues showed that among adolescents COC non-compliant behaviour was more pronounced (only 48% compliant) in inner-city teenagers attending a hospital clinic than among their suburban upper middle class counterparts (over 84% complaint with the COC) visiting a private clinic. Although several other patient characteristics have been associated with COC non-compliance (Rosenburgh et al. 1995a & 1995b), the relevance of these findings in other contraceptive use is open to question.

1.10 Detecting the fertile period of the cycle and ovulation

A simple and reliable means of ovulation detection will be invaluable in clinical practice as well as in research. In a clinical setting this information can be used by couples to time sexual intercourse in order to avoid or achieve pregnancy. Since the effects of sex steroids depend on the time of administration in the menstrual cycle, such information can assist in contraceptive research. An example would be the evaluation of the endometrial effects of peri-ovulatory administration of LNG.

The length of each menstrual cycle changes to a great extent in the same woman and also between women. The intra-subject and inter-subject variation of the length of the menstrual cycle is wide. This makes reliable prediction of the day of ovulation difficult. Estimates using clinical data have suggested the number of fertile days in each cycle to be around 6 days, ending on the probable day of ovulation (Wilcox et al. 1995). For years attempts have been made to identify potential markers of the fertile period, which would predict and detect ovulation. These include measurement of systemic changes that accompany the hormonal changes (basal body temperature (BBT) change & cervical mucus changes), assays of reproductive hormones in serum and urine, assays of cellular
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and secretory elements of endometrium, trans-vaginal sonographic imaging of ovarian follicular activity, and sonographic evaluation of the vascular and morphological changes in the uterus.

1.10.1 BBT change

BBT at rest shows a detectable shift of 0.2-0.4°C following ovulation due to the increasing production of progesterone from the corpus luteum. Hand-held electronic devices that utilise this temperature change to identify the end of fertile period have been tested claiming up to 94% agreement with the day of maximum follicular diameter as determined by ultrasound (Ismail et al. 1989; Flynn et al. 1991). However this method is neither prospective in estimating the day of ovulation, nor does it identify the onset of the fertile period.

1.10.2 Cervical-vaginal mucus or saliva changes

Under the influence of circulating sex steroids, the physical characteristics of the cervical mucus changes, and these changes are employed to prospectively identify the fertile period (WHO 1980) and to retrospectively estimate the day of ovulation (WHO 1983). NaCl in the saliva or cervico-vaginal mucus increases cyclically under the influence of oestrogens. This is responsible for the observed ‘ferning’ or crystallization pattern of these secretions that coincides with the fertile period in the cycle. A variety of small hand-held devices have been developed for the purpose of self-observing ferning patterns in either saliva or cervical- vaginal mucus. Small microscopes to determine the ferning in the context of other markers of fertility have been suggested with only limited success (Fehring et al. 1998).

Further attempts have been made to detect the cyclical volumetric changes in cervico-vaginal fluid (CVF), which correlate with levels of steroid hormones. By measuring the CVF volumes using a vaginal aspirator (Rovmeter) and correlating it with serial ultrasound measurements of the dominant follicle, Flynn et al. showed a consistent CVF
volume peak occurring the day before ovulation. They went on to demonstrate an abrupt fall in CVF volume following ovulation, which marked the onset of the infertile phase of the cycle (Flynn et al. 1988). Although the preliminary work suggests this an attractive, easy to use and inexpensive method of identifying the fertile period, further development and larger clinical studies are needed to substantiate these claims.

1.10.3 Hormone assays
It has been known for years that detecting the mid-cycle LH surge is the best marker of impending ovulation (WHO 1983). However, daily laboratory testing of serum or urinary LH did not offer a realistic means to be used in the community to detect ovulation in large scale. The subsequent development of enzyme-linked immuno-absorbent assay provided the answer with commercially available LH detection kits to test urine every 6, 12 or 24 hours (Garcia et al. 1981; Kasper et al. 1984; Hirsch et al. 1986; Vermesh et al. 1987). These were simple to use at home, and were rapid and reliable in detecting the mid-cycle urinary LH surge. However, the LH peak in urine precedes ovulation by approximately 24 - 48 hours (WHO 1980; Collins et al. 1996), hence if only LH was used to predict impending ovulation, the possible number of days of advanced warning achieved would be limited to two.

Further studies have investigated the possibility of incorporating information from rising levels of oestradiol that accompany the development of the dominant follicle and precede the LH surge, to make the warning of onset of the fertile period to be of more realistic length. Several authors identified a positive correlation between the early morning urinary levels of oestrone-3-glucuronide (E3G), the principal metabolite of oestradiol and the serum levels of oestradiol (Branch et al. 1982; Catalan et al. 1989). A rise of >50% of urinary E3G concentration over the mean value of the previous 3 days will forewarn the onset of the fertile period in between 83% - 90% of the ovulatory cycles (Aldercreutz 1982; WHO 1983; Schiphorst et al. 1985). Others have suggested
that it may be possible to use the urinary levels of progesterone metabolites to mark the end of the fertile period (Blackwell et al. 1998).

In 1999 Bonner and colleagues reported the development of a hand-held device (Personal system of Contraception or Persona®) as an aid to avoid sexual intercourse during the fertile period, and in 2000 Behre et al. described a similar system (ClearPlan® Fertility monitor) to be used for the opposite effect, i.e. to maximize the chance of conception by timing intercourse to the fertile period (Bonner et al. 1999; Behre et al. 2000). Both systems utilise information from E3G and LH levels in early morning urine to predict the fertile period and to detect ovulation, although they employ a different monitors, software and urine test sticks. It is fundamental that the algorithms utilised to warn of the fertile period in the two systems are different, since the contraceptive device requires a narrower margin of error and therefore indicates warning of potential fertility for at least 7 days (median 11, range 7 - 17 days), while the opposite is true for the pro-conceptive device in order to aid conception (median warning 6 days, range 0 - 20). The reported detection rate of LH peak (within 2 days of monitor indicated day of peak fertility) is around 91.1%.

1.10.4 Endometrial secretory elements
Biochemical tests of changing concentrations of cellular and secretory constituents of the endometrium that occur in response to ovarian hormones have been used to detect ovulation. Seif et al. reported the use of a monoclonal antibody to a cycle-dependent sialo-glycoprotein (Seif et al. 1989) to identify the peri-ovulatory period. Clearly, more studies in this direction are needed.

1.10.5 Ultra-sonography
For over two decades ultrasound has been used to monitor follicular growth and to identify ovulation (Queenan 1980; Vargyas, 1982). The reported ultra-sonic indices of ovulation include disappearance or sudden decrease of follicle size, appearance of
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ultrasonic echoes in the follicle, irregularity of follicular wall, and appearance of free fluid in the pouch of Douglas (Ecochard et al. 2000). Disappearance of the dominant follicular diameter appears to be the most sensitive of the indices with over 84% specificity and nearly 90% sensitivity (Vermesh et al. 1987; Behre et al. 2000; Ecochard et al. 2000). The urinary LH surge is also associated with ultrasonic changes in the uterus, including a continuous rise in endometrial thickness, and a gradual decrease in uterine artery pulsatility index (Bourne et al. 1996). However, the use of ultrasound is not suitable for home-use or for population based contraceptive studies.

1.11 Summary

Clinical research carried out to date suggests an urgent need for new methods of contraception and a need to explore the mechanisms of the existing contraceptives. Side effects such as unscheduled vaginal bleeding affect the acceptability of a contraceptive method and the detailed understanding of the mechanism of these side effects enables us to take appropriate steps to prevent them. The studies conducted as part of this Thesis aim to further the advancement in contraceptive research in such areas.

In order to explain the mechanism of post-coital contraceptive, we investigated the effect of LNG on the menstrual cycle and we also assessed the feasibility of using mifepristone as a once-a-month contraceptive pill.

Although compliance is a fundamental prerequisite for achieving the full potential efficacy of all drugs, including contraceptives, there is a dearth of information available on patient non-compliance with the use of different contraceptive methods. We sought to obtain insight into the compliant behaviour of women taking part in the contraceptive trial assessing the feasibility of administering once a month mifepristone.

The frequent and regular laboratory assays of steroid hormones in either blood or in urine have traditionally played a major role in research into female contraception. We
analysed the use of a personal fertility monitor as a reliable and more simple means to predict the potentially fertile period by monitoring frequently changing hormone levels.

To investigate the unexplained mechanism of mifepristone-induced endometrial shedding and vaginal bleeding, we evaluated the effects of mid-luteal phase administration of mifepristone on endometrial parameters.
CHAPTER 2

THE EFFECTS OF PERI-OVULATORY ADMINISTRATION OF LEVONORGESTREL ON THE MENSTRUAL CYCLE
2.1 Introduction
LNG 0.75 mg administered twice with the two doses 12 hours apart has been shown to be an effective method of EC when used within 72 hours of unprotected intercourse (Ho et al. 1993; WHO et al. 1998). Although the regimen is now licensed in the UK, USA and throughout much of Europe and is widely regarded as the EC method of choice (Guillebaud et al. 1998), the mechanism of action remains unknown. The mechanism of action of the Yuzpe regimen of emergency contraception (ethinyl estradiol 100 mcg and 0.5 mg LNG, two doses 12 hours apart, Yuzpe and Lancee 1977) is also incompletely understood but there is good evidence that it delays or inhibits ovulation in at least some cycles (Swahn et al. 1996; Croxatto et al. 2002). In the WHO study (1998) the efficacy of both LNG and the Yuzpe regimen decreased with time after intercourse (Piaggio et al. 1999) and both regimens had a similar effect on the timing of the subsequent menses, suggesting that the mechanism of action of the two regimens may be similar. It has also been shown that supra-physiological amounts of synthetic progestogens abolish the mid cycle LH surge leading to anovulation and delaying the onset of the subsequent menses (Kesseru et al. 1974; Landgren et al. 1990; Craft et al. 1975; Sopna et al. 1974; also refer Table 1.2).

To test the hypothesis that it acts as a post-coital agent by abolishing the pre-ovulatory LH surge and by delaying ovulation, we administered LNG 0.75 mg twice to 12 healthy female volunteers in the fertile period (immediately before ovulation) of the menstrual cycle and investigated the effects on the timing of ovulation and of the next menses; bleeding patterns; ovarian activity and LH concentrations.

2.2 Materials & Methods
This was a prospective, randomised, double blind, cross-over study undertaken in one centre. Twelve healthy women (mean age 33 (range 26 - 41 years)) with regular cycles (mean 27.63 days (range 25 – 30 days) and mean BMI 25.71 (range 20 - 34)) were recruited. They were all using a reliable non-hormonal method of contraception or were abstinent during the study. All subjects gave written informed consent to
participation in the study, which was approved by the Lothian Research Ethics Committee.

A method was sought to provide a convenient means of identifying the fertile period prior to ovulation, and thereby to time the administration of LNG. A home-use fertility monitor was used for this purpose. This product is similar to the Personal System of Contraception (Persona, Unipath Bedford UK) (Bonnar et al. 1999) in that it monitors E3G and LH in urine, but differs in that it has been specifically designed for maximising the chance of conception. Unipath Diagnostics Co., Princeton, NJ, markets the fertility monitor in the US and Unipath Ltd., Bedford, UK markets it in Britain as ClearPlan Easy Fertility Monitor (CPEFM) (Figure 2.1).

**Figure 2.1** *ClearPlan Easy Fertility Monitor with a urinary test stick*  
(LCD on the monitor shows peak fertility).

The system comprises a hand-held monitor and disposable dual-assay urine test sticks, and is used to simultaneously detect LH and E3G levels in early morning urine. The monitor optically measures the intensity of the lines that form on the test sticks after sampling, and the system will delineate three levels of fertility (Low, High and Peak fertility) according to the optical signal changes detected. Low Fertility will be displayed from day 1 of the menstrual cycle, until the hormone levels rise above the baseline levels. A change from Low to High Fertility is triggered by
detection of elevated E3G levels. The change from High to Peak Fertility is triggered by the detection of an LH surge. Peak fertility is displayed on the day of the LH surge and on the following day before returning to low fertility.

Each woman was studied during four cycles, and was issued with a monitor at the beginning of the study. Subjects were asked to use the CPEFM according to instructions, and familiarised themselves with the monitors by using it during a pre-study cycle to identify the days of high fertility and the day of the LH surge. They also recorded days of vaginal bleeding.

Data from the pre-study cycle was used to predict the timing of the LH surge and of the fertile phase during the study cycles and thereby to predict when treatment should be administered. The three study cycles followed immediately after the pre-study cycle. Six subjects were randomly assigned to treatment arm A and received LNG in the first study cycle and placebo in the third study cycle. The remaining six subjects were randomised to treatment arm B and received placebo in the first study cycle and LNG in the third study cycle. The second study cycle was a “washout” phase for all women during which time they also received placebo tablets (Figure 2.2).

The randomisation list was produced using SPSS Rv.Bernouilli function such that each study number was randomly assigned to either treatment arm A or treatment arm B with the same probability i.e. 0.5.

LNG and placebo were visually identical and were pre-packed. Each subject collected a sample of early morning urine daily from the first day of the first study cycle until and including the first day of the menstrual bleed signalling the end of the last study cycle. Samples were frozen and later assayed in batches (with all samples from one subject assayed in a single batch) for measurement of urinary LH, E3G and pregnanediol-3-glucuronide (P3G).
Figure 2.2 Study design

Quantitative assessment of urinary LH was performed using a LH MAIAclone kit (BIOSTAT-DIAGNOSTICS, Stockport, Cheshire, UK). This method incorporates two high affinity monoclonal antibodies into an immunoradiometric assay system and offers a working range of 1.5 – 200 mIU/ml. Urinary P3G was measured using a direct enzyme immunoassay sensitive (working range 0.16 ug/L – 20 ug/L), while direct immunoassay was used to measure E3G levels (working range 8.36 nmol/L - 2140 nmol/L). Intra-assay coefficients of variation were 6% for E3G, 10% for P3G1 and 3% for LH (Yong et al. 1992). Geometric means of daily replicates were divided by the respective daily creatinine concentration to correct for variations in the dilution of the urine specimen.

During study cycles 1 and 2 women were asked to take the study medication on the first day of high fertility as identified by the monitor. However, by the third study cycle, the variation in the number of High Fertile days (range 0-8 days) meant that the monitor could not be used to administer medication on LH-2 in every cycle. Therefore, we had to adopt a different method of calculating the anticipated day of the LH peak for each cycle based on the monitor information from the previous cycles (including the pre-study cycle). Hence, in the third study cycle, the medication was taken two days prior to the anticipated day of the LH peak. In all cycles the first tablet was taken at 11.00 hours and the second at 23.00 hours. A sample of venous blood was collected 5 – 7 days after treatment, stored and later assayed for
progesterone using Coat-A-Cont solid-phase radio-immunoassay. The subjects kept a
daily record of all vaginal bleeding experienced during the 4 cycles, the fertility
status information displayed each day on the monitor LCD and the days on which the
study medications were taken.

2.2.3 Statistical Analysis
We calculated that a total of six subjects in each of the two treatment arms would
give more than 90% power to detect a delay of menses of \( \geq 5 \) days in 95% of cycles.
The sample size calculation indicated that only 2 subjects per treatment order arm are
required for 90% power if we assumed that LNG delayed (\( \geq 5 \) days) the subsequent
menses in 95% of cycles and the menses would be delayed in 5% of cycles after
placebo administration. Since greater numbers were required for the reliability of
estimates and to take account of the fact that the menstrual cycles of subjects may
have been less regular during the study than had been indicated at the recruitment
stage, we recruited 12 subjects.

Preliminary analysis was performed to determine whether parametric tests were
appropriate for analysis of the data. Outlying data points were investigated while the
treatment was still blinded. The period effect and interaction between treatment and
period effect were tested (two sample t-test) before progressing to testing of a
treatment effect and was non-significant. Comparisons between LNG versus placebo
cycles, was tested by paired t-test. Since the data appeared to be skewed a non-
parametric Mann-Whitney U test was performed to compare the difference between
urinary E3G levels on the day of LNG treatment in cycles that had delayed LH peak
and the 8 cycles that did not.

For the purpose of the study the following definitions based on the quantitative data
were created.

A significant delay in the onset of next menses: Delay of 5 or more days from the
expected onset of menses (based on the mean cycle length for the 2 placebo cycles).
The LH peak was defined as a significant rise in urinary LH concentration, with a minimum of 50% rise above the average baseline level for 4 preceding days and which remained elevated for a minimum of 3 days.

The first day of the LH peak was defined as the day of the first significant rise (>50% above the baseline) seen at the beginning of the LH peak.

Retrospectively predicted first day of the LH peak for the treatment cycles was the calculated mean of the first day of LH peak in the two placebo cycles.

A significant delay in the first day of the LH peak: Delay of 5 or more days from the expected first day of the LH peak in the treatment cycle (based on the mean first day of the LH peak during the 2 placebo cycles).

Follicular phase: time from the first day of the menses until the day of the first significant rise in urinary LH (LH+0) inclusive.

Luteal phase: time from the day after the first day of the urinary LH peak (LH+1) until, and including, the day before the first day of the next menses.

2.4 Results

A total of 48 menstrual cycles were studied - 12 pre-study cycles and 36 study cycles. Data from daily urine samples were available for 34 out of the 36 study cycles. In one woman (S103) the first study cycle, which was a placebo cycle, was prolonged (41 days) as a consequence of a delay in ovulation. Her usual cycle length was 25 days, this cycle was excluded from the analysis. In a second woman (S110) there were no daily urine samples available from the washout cycle as she was abroad on holiday. Therefore, daily urine samples were only available for this subject from two study cycles (the treatment cycle and one placebo cycle).

2.4.1 Timing of administration of LNG

Six women took LNG in the first study cycle and six took it during the third study cycle. During the first study cycle, 10 women took the tablet (either placebo or LNG) on the first day of high fertility as indicated by the monitor. The remaining two women took the tablet on the first day of the urinary LH peak because the monitor failed to identify any High fertile days prior to the LH peak.
The variation in the number of High fertile days (0-8 days) declared by the monitor meant that the system could not be used to predict LH-2 in every cycle. Therefore for cycle three we calculated the anticipated first day of the urinary LH peak from the information gathered from the pre-study cycle and study cycles 1 and 2 for each woman and instructed subjects to take the tablet two days before the anticipated day of the LH peak.

After completion of the study, we retrospectively calculated the predicted first day of the urinary LH peak for every treatment cycle based on the mean of the first day of LH peak in the two placebo cycles and the pre-study cycle. When we applied this retrospectively predicted definition to all 12 treatment cycles, the day of taking LNG ranged from six days before until one day after the first day of the anticipated LH peak. However, in reality, LNG was never taken after the first significant rise in urinary LH concentrations in any treatment cycle. The timing of the LH peak in each of the four cycles, the predicted day of the LH peak day and the timing of LNG and placebo treatment in relation to the start of the actual LH peak are shown in table 2.1.

2.4.2 Cycle length
Treatment with LNG in the pre-ovulatory period significantly prolonged (by 5 days or more) the mean cycle length in four women (33 % of the sample, Table 2.2). All four women reported vaginal spotting two to three days after taking LNG and they all had a second episode of vaginal bleeding between 9 and 16 days after the delayed LH peak. In the remaining eight women there was no significant difference in cycle length between treatment and placebo cycles. One of this group of women however, reported light vaginal bleeding starting a week after taking LNG, the bleeding continued until she started what she regarded as a normal menstrual period which followed a fall in urinary pregnanediol levels. Her hormone profile during the treatment cycle follows a normal pattern. The four women, who had prolonged cycles, took LNG on a day when urinary E3G was at a low level when compared to the remaining 8 women but this difference did not reach significance (8 vs. 19.16 mmol/mol, p = 0.12).
Table 2.1. Timing of LH peak, predicted day of the LH peak and timing of LNG in treatment cycles for all subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Day of the LH peak (LH &gt;50%)</th>
<th>Predicted day of the LH peak in the treatment cycle</th>
<th>Timing of LNG in relation to the day of the LH peak in the treatment cycle</th>
<th>Timing of LNG in relation to the predicted day of the LH peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PSC</td>
<td>Placebo</td>
<td>Placebo (washout)</td>
<td>Treatment</td>
</tr>
<tr>
<td>S101</td>
<td>12</td>
<td>13</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>S102</td>
<td>M</td>
<td>20</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>S103</td>
<td>12</td>
<td>27**</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>S104</td>
<td>M</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>S107</td>
<td>17</td>
<td>14</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>S111</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>S112</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>S105++</td>
<td>14</td>
<td>13</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>S106*</td>
<td>14</td>
<td>14</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>S108*</td>
<td>17</td>
<td>18</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>S109*</td>
<td>14</td>
<td>10</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>S110*</td>
<td>16</td>
<td>14</td>
<td>££</td>
<td>40</td>
</tr>
</tbody>
</table>

++ The woman with normal LH peak but no significant rise in pregnanediol in the luteal phase = anovulatory cycle.

M = LH peak missed by the CPFM

**= excluded, unusually delayed ovulation

££ = excluded, no daily urine available

* Women who had a delay of the LH peak by >5 days.
**Table 2.2**  
*Mean length of placebo and treatment cycles*

<table>
<thead>
<tr>
<th>Mean cycle length</th>
<th>Treatment cycles</th>
<th>Placebo cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cycles, n = 12&lt;sup&gt;1&lt;/sup&gt;</td>
<td>32.17 (SD +/-3.36)</td>
<td>26.33 (SD +/- .42)</td>
</tr>
<tr>
<td>N = 4, Delay of &gt; 5 days&lt;sup&gt;2&lt;/sup&gt;</td>
<td>42.75 (SD +/-8.42)</td>
<td>27.13 (SD +/-1.84)</td>
</tr>
<tr>
<td>N = 8, remaining cycles&lt;sup&gt;3&lt;/sup&gt;</td>
<td>24.88 (SD +/-2.1)</td>
<td>26.13 (SD +/-1.69)</td>
</tr>
</tbody>
</table>

1. p = 0.14, 2. p = 0.04, 3. p = 0.12

### 2.4.3 The first day of the LH peak.

In the four women with long cycles, LNG appeared to abort the LH peak and a subsequent LH peak occurred 7 to 16 days later followed by a normal rise in urinary pregnanediol. The urinary hormone profile of one woman (S110) is illustrated in charts 2.1a and 2.1b. In the remaining eight women, LNG did not affect the timing of the LH peak when taken immediately before ovulation. Chart 2.3 illustrates the hormone profile in one of these women (S101).
Charts 2.1a & 2.1b
Daily levels of LH (●), E3G (Δ), and pregnanediol (○), in urine relative to the cycle day.

2.1a Placebo cycle of a woman (S110).
2.1b Treatment cycle of the same woman (S110) showing significantly prolonged cycle following pre-ovulatory LNG.

↓ Day of taking LNG or placebo tablet       Day of the LH surge
Charts 2.2 and 2.3
Daily levels of LH (●), E3G (Δ), and pregnanediol (○), in urine during the treatment cycles,
2.2 Of the woman (S105) with no significant rise in pregnanediol following LNG
2.3 Of a woman (S101) with apparently normal cycle length

Day of taking LNG Day of the LH surge in the placebo cycle

Day of the LH surge
2.4.4 Length of the luteal phase

In all twelve volunteers the luteal phase was significantly shortened following treatment with LNG as compared with the placebo cycles (mean length 11.5 days (SD +/-1.8) vs. 12.9 days (SD +/-2.5) p = 0.005, Table 2.3).

Table 2.3 Length of the luteal phase (n = 12)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment cycle</th>
<th>Mean for Placebo cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>12</td>
<td>13.5</td>
</tr>
<tr>
<td>102</td>
<td>8</td>
<td>8.5</td>
</tr>
<tr>
<td>103</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>104</td>
<td>13</td>
<td>15.5</td>
</tr>
<tr>
<td>107</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>111</td>
<td>12</td>
<td>13.5</td>
</tr>
<tr>
<td>112</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>105 ++</td>
<td>11</td>
<td>13.5</td>
</tr>
<tr>
<td>106 *</td>
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</tr>
<tr>
<td>108 *</td>
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<td>11.5</td>
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<tr>
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<td>13</td>
<td>16.5</td>
</tr>
<tr>
<td>110 *</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

++ The woman with normal LH peak but no significant rise in pregnanediol in the luteal phase = anovulatory cycle.
* Women who had a delay of the LH peak by >5 days.

2.4.5 The effect on total LH during the luteal phase

Daily urinary LH concentrations were summated from the first day of the LH peak (LH +0) up to the day before the first day of the next menses to give a value for total LH concentrations. The eight women in whom pre-ovulatory LNG did not affect the cycle length, showed a significant (p = 0.01) decrease in total LH in the treatment cycles (18.66 U/mol creatinine (SD +/-8.9)) as compared with the placebo cycles (27.08 U/mol creatinine (SD +/-13.57)). In contrast among the four women with significantly longer cycles after taking LNG, there was no difference in the total LH secretion in the luteal phase (mean total LH = 18.83 U/mol creatinine (SD +/-8.3))
versus mean total LH for placebo cycles of 17.75 U/mol creatinine (SD +/-2.8) p = 0.81).

2.4.6 Effect on pregnanediol in the luteal phase
Comparing the sum of daily pregnanediol concentrations in the luteal phase (from LH +1 onwards up to the day before the first day of next menses) during the treatment and placebo cycles was employed to indirectly assess corpus luteal function.

In one woman (S105) after taking LNG, there was no significant rise in urinary pregnanediol levels (>0.5 mmol/mol creatinine as expected in the mid-luteal phase) despite an apparently normal LH peak. The mid-luteal serum progesterone level during this treatment cycle was consistent with an anovulation (<5 nmol/L) (chart 2.2).

In the remaining 11 subjects the total values of pregnanediol did not show a significant difference between placebo or treatment cycles.

2.5 Discussion
Evaluation of daily hormone concentrations confirmed that all twelve women in our study took LNG before the LH peak, and before ovulation.

Seven women had apparently normal ovulatory cycles after taking LNG. Five of them took LNG on the day before the LH surge and one on the day of the surge. It is possible that the timing of LNG in these women was “too late” to influence an event already well underway. Recently, others have shown a similar effect when LNG is taken immediately prior to the LH surge, late in the follicular phase (Durand et al. 2001).

In four women (25%), LNG aborted the LH peak, delayed the ovulation and lengthened the cycle. All four women took LNG 2 to 4 days before the predicted LH
surge (based on the two placebo cycles). One woman did not ovulate at all despite having an LH surge 2 days after taking LNG.

It is apparent that different women respond differently to the administration of LNG. The effects observed may be related to administering LNG at a specific stage in follicular development. Even though the timing of the LNG in relationship to the onset of the LH surge did not appear to be different between women in whom ovulation was affected and those in whom it was not, it would be naïve to accept that our predicted day of the LH peak based on information gathered in two cycles was always accurate. In fact, the four women with delayed ovulation had apparently low urinary E3G on the day the treatment was administered. This suggests that they took LNG at an earlier stage of follicular maturation and a similar effect was not seen in the others who took LNG at a more advanced stage of follicular development. A more detailed study by Duran et al. also suggested that LNG in mid follicular phase suppress follicular maturation and secretion of E2 and P4, and thereby inhibit LH surge and ovulation (Duran et al. 2001). When administered immediately prior to the onset of the LH surge, LNG did not affect the LH surge, ovulation or the cycle length in their study. They employed daily serum levels of gonadotrophins and steroid hormones and ultrasound scans to correlate follicular size and maturity to the time the administration of LNG. In agreement with this explanation, another recent study has also shown that when LNG was administered 2 days before the anticipated LH surge, the ovulation was not affected (Marions et al. 2002).

The available literature suggests a direct suppression of pre-ovulatory E2 rise by progestins (Schreiber et al. 1980; Goodman et al. 1982; Fortune et al. 1983; Weibe et al. 1984) and that may be the mechanism by which LNG inhibits the LH surge.

Many have reported a shorter luteal phase, after peri-ovulatory administration of LNG (Johansson et al. 1971; Sopna et al. 1975; Craft et al. 1975; Landgren et al. 1989; Duran 2001). In our study, the seven women who apparently ovulated normally had a reduced total luteal LH and a shortened luteal phase. Basal levels of LH are
essential for the normal secretory function of the corpus luteum (Vande Wiele et al. 1970). In the mid-luteal phase LH inhibition by the administration of GnRH antagonists consistently resulted in luteolysis in women as well as in non-human primates (Hutchison et al. 1984; Hall et al. 1991; Ravindranath et al. 1992). There are no direct ways of measuring the corpus luteal function. Although we did not find a significant difference in the urinary pregnanediol levels after LNG, it is possible that the shortened luteal phase observed was a consequence of reduced total LH and may have a contra-gestive effect.

If LNG acts as an emergency contraceptive only by interfering with ovulation, the expected efficacy should fall below 42% (5 out of 12 women). Ho and colleagues reported that LNG reduced approximately 60% of the expected number of pregnancies (estimates were based on the table of probabilities of pregnancy at different cycle days by Dixon et al. 1980). LNG fared better in the WHO study (WHO 1998) with overall 85% reduction of expected number of pregnancies (the analysis of the prevented fraction was based on the modified Wilcoxon estimates of conception probabilities (Trussell et al. 1998)).

Both studies reported effectiveness against estimates based on historical data. The fertile period was determined on the assumption that ovulation occurred 14 days before the next expected menses. The validity of using these estimates directly relies on the accuracy of reported menstrual cycle data. Women do not always record their menses, and the sexual intercourse responsible for requesting EC maybe unpremeditated. Reporting errors are common and the estimates can be inaccurate. In addition, other factors such as biological variability of the day of the ovulation and the length of the luteal phase, factors affecting the probability of pregnancy unrelated to the timing of intercourse, and heterogeneity among couples in fecundability can distort the estimated number of pregnancies. In a study comparing the efficacy of the Yuzpe regimen of EC with a single dose of mifepristone (Glasier et al. 1992), there were frequent discrepancies among subjects between the stage of the cycle as estimated from the date of the LMP and that suggested by circulating concentrations
of progesterone. In a recent report, Sterling & Glasier went on to confirm their previous suggestions, and they showed that the date of LMP, timing of intercourse, and the number of acts of intercourse in the same cycle were inaccurately recalled by a significant proportion of women requesting EC (Sterling & Glasier 2002). There has never been a placebo-controlled trial of EC. Thus, it is possible that the genuine effectiveness of LNG as an EC is less than 42%.

On the other hand, the discrepancy noted in the estimated effectiveness of LNG and the prevalence of ovulation delay or inhibition in our study may be due to mechanisms of action other than interference with ovulation. Our study was not designed to investigate the other possible mechanisms by which LNG works. However, one woman in our study reported slight vaginal bleeding after taking LNG with an apparently normal LH peak, cycle length and hormone profile. This may suggest an additional effect of LNG on the endometrium. Landgren and colleagues (Landgren et al. 1989) showed that follicular phase administration of multiple doses of 0.75 mg LNG suppressed the proliferative activity of the endometrium, while no significant endometrial effect was seen if it was taken in the luteal phase. Glandular atrophy, stromal decidualisation and epithelial cell inactivation has been observed after chronic local exposure of the endometrium to LNG (Pakarinen et al. 1995). Locally administered LNG also induces expression of the potent contraceptive glycoprotein Glycodelin A during the implantation period (Mandelin et al. 1997). In high doses (20 – 40 ng/ml) progesterone inhibits the growth of human capillary endothelial cells in culture (Peck et al. 1995). Therefore, the question remains as to whether similar alterations occur in the endometrium after taking the EC regimen of LNG, and whether these changes are sufficient to prevent implantation and account for the observed contraceptive efficacy of LNG. The two recent studies, which examined the effect of peri-ovulatory phase administration of LNG on endometrium, did not report any significant morphological changes (Duran et al. 2001; Marions et al. 2002).
The effect of progestogens on cervical mucus and on the cervix is well documented and this is thought to be the main mechanism by which the progestogen-only pill exerts its anti-fertility action (Odeblad et al. 1972; Daunter et al. 1976; Moghissi et al. 1973). However, even if LNG has an effect on cervical mucus, which interferes with sperm penetration, that action is unlikely to prevent pregnancy when taken some 12 - 72 hours after coitus.

Progesterone stimulates a rapid influx of calcium into the spermatozoon via a non-genomic action (Tesarik et al. 1996; Aitken et al. 1996; Blackmore et al. 1996) and induces an acrosomal reaction (Oehninger et al. 1994). An impaired response of the spermatozoon to progesterone is a clinically well-recognised defect in the infertile male (Falsetti et al. 1993). Given their total lack of transcriptional ability, it would appear that in the sperm the non-genomic progesterone receptor would have to be responsible if LNG were to have an EC effect.

One woman (S103) in our study showed a delayed LH peak (on day 27) during a placebo cycle and subsequently the length of that cycle was prolonged to 41 days (her usual cycle length was 25 days). In contrast to the four women who experienced similar prolongation of the cycles after taking LNG, this woman (S103) did not report any inter-menstrual vaginal bleeding. Although we excluded this cycle from our analysis, similar spontaneously occurring prolonged cycles (with delayed ovulation) can influence the results of studies into EC.

The fertility monitor is a product designed to indicate the potentially fertile days leading up to ovulation, through the detection of a significant rise in the levels of the urinary oestrogen metabolite E3G. The position of this rise relative to the time of ovulation will vary between women, and therefore the monitor will not consistently provide two days warning of the LH surge in every cycle. It was understood that no home use method could consistently provide such focussed warning of ovulation, but the monitor was considered to represent the best available home use technology for ovulation prediction.
The reason for using the monitor to time the administration of LNG or placebo also to avoid having to subject the volunteers to regular blood samples and ultrasound scans.

However, due to the variability in the number of high fertile days declared prior to the LH surge, greater reliance had to be placed on calendar calculations to predict the LH surge.

In conclusion, we suggest that LNG taken immediately before ovulation acts as an EC by delaying or preventing ovulation. Other plausible actions of LNG including the retardation of the endometrium, interfering with sperm motility and altering cervical mucus may be important, and need to be explored further.
CHAPTER 3

FEASIBILITY OF ADMINISTERING MIFEPRISTONE AS A ONCE-A-MONTH CONTRACEPTIVE PILL
3.1 Introduction

While hormonal contraception is extremely popular (it is used by almost 100 million women world-wide) many women continue to be deterred from using it because of perceived risks to health such as breast cancer and due to side effects such as weight gain. Most of the risks and the side effects are the results of prolonged exposure to steroids and many women, in a variety of cultural settings, find the idea of a pill, which they need take only once each month, an attractive concept (Rimmer et al. 1992; Glasier et al. 1999).

Progesterone is essential for the establishment and maintenance of human pregnancy. The anti-progesterone mifepristone is a synthetic 19-norsteroid, which acts by blocking the action of progesterone at the receptor level (Spitz & Bardin 1993), and thus, having multiple potential anti-fertility actions. When administered in the early luteal phase mifepristone retards endometrial development, without disturbing the timing of menses (Swahn et al. 1988; Berthois et al. 1991; Mentausta et al. 1993). It also changes uterine contractility to a pattern more usually seen in the late luteal phase (Gemzell-Danielsson et al. 1990). In 1993 Gemzell-Danielsson and colleagues conducted a pilot study in which a single dose of 200 mg of mifepristone was given in the early luteal phase (2 days following the peak of the LH in urine). Out of 124 cycles in which coitus took place during the fertile period, they observed only 1 pregnancy. There was no disruption of the timing of the subsequent menstrual bleed, although in 32% of the cycles slight vaginal bleeding was reported 2 – 3 days after treatment.

The main problem in developing a once-a-month contraceptive is finding a method that both reliably and easily identifies the start of the LH peak. Gemzell-Danielsson tried to solve this problem by using the LH sticks for home urine testing (Ovu-quick, Organon). In their study 12 out of 169 cycles were deemed to be anovulatory. However, it is not possible to determine if the LH peak truly was absent, or if the method failed to detect a surge. The woman may have read the test result wrongly or even forgot to perform a test
on the appropriate day. Moreover, anovulatory cycles would not be expected to exceed 5 per cent among healthy women in that age group (18–35 years) (Vollman et al. 1977).

Unipath (Bedford, UK) have developed a Fertility Awareness Monitor, which simultaneously detects LH and E3G in a sample of early morning urine. The monitor utilises the information from both of these parameters to determine the day of the LH peak. The time from first significant rise of LH in the urine to ovulation is reported to be around 24–48 hours (Collins et al. 1996). The monitor thus, should provide a convenient method of identifying the early luteal phase. In addition, it provides a 6-month record of the actual signal levels corresponding to daily hormone levels in urine, and of the days on which tests were performed.

We investigated the use of the monitor to identify the LH surge in a group of women who used 200 mg of mifepristone on day LH +2 as their sole method of contraception.

3.2 Subjects and Methods

3.2.1 Subjects

This was a single centre study in healthy female volunteers, approved by the Lothian research ethics committee. All subjects gave written informed consent to participation. Fifty two sexually active women, with regular (25–32 day) menstrual cycles were recruited from a large Family Planning Clinic in Edinburgh. If any women had a significant medical condition or if they or their partners had a history of fertility problems, they were excluded from the study.

3.2.1.1 Treatment group

Thirty two women were recruited to the treatment group. None had been taking hormonal preparations within the 2 months prior to the start of the study and all had at least 2 spontaneous menstrual periods since stopping hormonal contraception. All women underwent screening at the time of recruitment including routine physical and
gynaecological examination. A venous blood sample was taken for full blood count, serum biochemistry and liver function. The study period was from day 1 of the menstrual period following screening, and lasted for up to seven consecutive menstrual cycles in which subjects took 200 mg mifepristone once per month.

### 3.2.1.2 Control group

The control group consisted of 20 healthy women with regular menstrual cycles who were trying to become pregnant (for less than 6 months prior to the enrolment into the study) and hence, were not using contraception. They were provided with a monitor, which they used according to the manufacturer’s instructions. Women were advised that their chance of conception would be higher if they were to have sexual intercourse during the fertile period, identified by the monitor. The controls took part in the study until pregnancy occurred or for a maximum of 6 cycles if they did not conceive.

### 3.2.2 Method

All subjects and controls were provided with a home use hormone monitoring system (Unipath, Bedford, UK). The system comprises a hand-held monitor and disposable dual-assay urine test sticks, and is used to simultaneously detect LH and E3G levels in early morning urine (Figure 2.1). The monitor optically measures the intensity of the lines that form on the test sticks after sampling, and the system will delineate three levels of fertility (Low, High and Peak fertility) according to the optical signal changes detected. Low fertility will be displayed from day 1 of the cycle, until the hormone levels rise above the baseline levels. A change from Low to High fertility is triggered by detection of elevated E3G levels. The change from High to Peak fertility is triggered by the detection of an LH surge.

Peak fertility is displayed on the day of the LH surge and on the following day. Subsequently High fertility is displayed for one day prior to a return to Low fertility. At the start of each menses, the subjects pressed the ‘m’ button on their monitor to initiate
that cycle of use, at a time suitable for testing the first urine of the day. For the rest of the month, the subjects were required to consult the monitor display each morning (3 hours either side of the time when 'm' button was set) to determine whether they needed to perform a test that day. Outwith this six hour time window the monitor would not accept a test. The system requests one test every day for up to a total of 10 or 20 tests, depending on the length of the woman’s cycle, and the timing of her LH surge. Embedded software within the monitor collects and analyses data from each cycle to identify and display fertility status to the user, and stores data for several months (Figure 3.2 & 3.3). This product is similar to the Personal System of Contraception (Persona, Unipath Bedford UK) (Bonnar et al. 1999) in that it monitors E3G and LH in urine, but differs in that it has been specifically designed for maximising the chance of conception. Unipath Diagnostics Co., Princeton, NJ, markets the fertility monitor in the US and Unipath Ltd., Bedford, UK markets it in Britain as Clearplan Easy Fertility Monitor (CPEFM) (Figure 2.1).

Mifepristone was taken two days after the day of the first day of Peak Fertility (LH surge). With each cycle, subjects followed the same protocol, and were reviewed by the investigator monthly, on day LH +2. Just before taking the 200 mg tablet of mifepristone, a venous blood sample was taken, and later assayed for progesterone. At the beginning of the study, if the LH surge was not identified by day 21 of the cycle, the subject was instructed to continue testing, but mifepristone was not given in that cycle. The subject was also advised to use barrier contraception from day 21 until the onset of the next menses. After the second pregnancy (which occurred due to a failure in detecting an LH surge), we changed this practice. We calculated the estimated day of LH surge for each month based on information from the previous cycles. If the women did not detect an LH surge either within 3 days after the anticipated day of LH surge or by day 19, a blood sample was taken for rapid serum progesterone assay. If the progesterone level was >5nmol/L and if the woman was at risk of pregnancy, mifepristone was administered. All subjects and controls kept a menstrual record card,
recording all vaginal bleeding experienced during the study and the days on which they had sexual intercourse.

**Figure 3.2  Information retrieved from the Fertility monitor-1**

This figure illustrates the monitor display as seen by the volunteer each day. Empty box = low fertility, Half filled box = high fertility, Completely filled box + egg = peak fertility. Underneath each box, a solid line represents a correctly performed test while a missed / inaccurately performed test is represented by a broken line. Volunteer using this particular monitor showed perfect compliance.

Cycle: 1, Start: 22 February 1999, Length: 29 days

Cycle: 2, Start: 23 March 1999, Length: 27 days

Cycle: 3, Start: 19 April 1999, Length: 28 days

Cycle: 4, Start: 17 May 1999, Length: 32 days

Cycle: 5, Start: 18 June 1999, Length: 30 days

Cycle: 6, Start: 18 July 1999, Incomplete Cycle
Figure 3.3  *Information retrieved from the Fertility monitor-II*

This figure illustrates the additional information that can be stored and retrieved from the monitor on the details of each test. The closed circles represent the LH signal, and the open squares represent the E3G signal. The solid green line indicates the LH peak. Fertility status is indicated as L = low fertility, H = high fertility, & P = peak fertility.

Subjects also marked the first day of the Peak fertility as identified by the monitor and the day of taking the study medication.

If menstruation was overdue by more than one week the investigator performed a pregnancy test. Provided this was negative, the subject continued in the study and the next cycle was deemed to start with the onset of menses. Since the effect of mifepristone
taken in very early pregnancy is unknown, and teratogenic effects could not be ruled out, women who would not consider terminating any pregnancy were not recruited to the treatment group.

At the end of the study, the subjects attended for a final visit, when a routine physical and gynaecological examination was performed. Full blood count, serum biochemistry and liver function were reassessed.

The following definitions were created for the purpose of the study.

**Imperfect Use** was defined as failure to detect an LH surge through performing the test incorrectly (e.g.; dipping a test stick in urine 30 or more minutes before it being read by the monitor), or failing to perform tests as requested by the monitor.

**Monitor method failures** were defined as failure to detect an LH surge despite performing all tests as requested.

**High fertile days**: days preceding the urinary LH surge as indicated by the monitor to be potentially fertile.

**Peak fertile days**: The first day of a significant rise in urinary LH detected by the monitor, and the following day.

**The Fertile period** of the cycle was defined as three days before until two days after the urinary LH surge (LH -3 to LH +2).

**Exposure cycles** were cycles in which women reported having sexual intercourse at least once during the fertile period.

**3.2.3 Statistical analysis**

Cycle lengths and serum progesterone concentrations were compared by two-sample t-tests. Confidence limits for efficacy were derived from confidence limits for relative risk calculated by the Greenland and Robins method (Greenland & Robins 1985).
3.3 Results

Table 3.1 shows the demographic characteristics of the women who took part in the study. The women in treatment group were slightly younger (mean age 30 years) than those in the control group (mean age 32.92 years). Otherwise there were no differences between subjects and controls.

<table>
<thead>
<tr>
<th></th>
<th>Treatment Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 32</td>
<td>N = 20</td>
</tr>
<tr>
<td>Age</td>
<td>Range 18 – 39</td>
<td>26 – 40</td>
</tr>
<tr>
<td></td>
<td>Mean (+/- SD) 30 (+/-5.4)</td>
<td>32.9 (+/-4.5)</td>
</tr>
<tr>
<td>BMI</td>
<td>Range 19 – 38</td>
<td>21 – 29</td>
</tr>
<tr>
<td></td>
<td>Mean (+/- SD) 23.6 (+/-4.3)</td>
<td>23.8 (+/-2.7)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7 (21.9)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Non-smokers (%)</td>
<td>21 (65.6)</td>
<td>16 (80)</td>
</tr>
<tr>
<td>Ex-smokers (%)</td>
<td>4 (12.5)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Previous pregnancies 1+ (%)</td>
<td>19 (59.4)</td>
<td>14 (70)</td>
</tr>
<tr>
<td>never been pregnant (%)</td>
<td>13 (40.6)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Ever abortion (%)</td>
<td>15 (46.9)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Married / Co-habiting (%)</td>
<td>28 (87.5)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Single (with a regular boy friend) (%)</td>
<td>4 (12.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

3.3.1 The probability of pregnancy in the control group

20 women were recruited to the control group and three withdrew before completing the study. Two withdrew from the study as they found using the system “too stressful” and one withdrew because she no longer wished to plan a pregnancy. Data were collected from 50 control cycles during which 12 pregnancies occurred. Average frequency of intercourse was 1.7 episodes per week in the 39 control cycles in which the women kept a record of their sexual activity. In 37 cycles women had intercourse at least once during
the fertile period. In two cycles intercourse did not occur during the fertile period, while in 11 cycles the exposure status was unknown, as women failed to keep a record of sexual activity. Eight pregnancies occurred in the first exposure cycle.

If we assume that all 11 cycles from which information on sexual activity was lacking were exposure cycles, the probability of pregnancy was 0.25. However if those cycles were all non-exposure cycles, the probability of conception would be 0.32. Therefore among the control group the overall probability of pregnancy if sexual intercourse took place at least once during the fertile period lies between 0.25 – 0.32.

### 3.3.2 Contraceptive efficacy of the method

Thirty two volunteers were treated with a single dose of 200 mg of mifepristone administered in luteal phase of the cycle as their sole method of contraception between one and seven cycles. They contributed a total of 178 cycles, and in 167 cycles mifepristone was administered. Eight women withdrew from the study before completion, two women moved out of the area, three ended their relationship, two conceived during the study and one lost confidence in the method.

Two clinical pregnancies occurred in the 178 cycles studied. The first pregnancy was a true treatment failure, which occurred in a woman (para 1) who took mifepristone on day 14 (LH +2) of her first treatment cycle. She opted for a surgical termination of pregnancy, which was performed at eight weeks of gestation (confirmed by ultrasound scanning). In the second woman (para 3), an LH surge was not identified in her third study cycle hence she did not receive treatment with mifepristone (Figure 3.4), menses did not occur and on day 37 after her last menstrual period an ectopic pregnancy was diagnosed and treated surgically. In a third woman a biochemical pregnancy was diagnosed (serum β hCG only rising to 34 U/L), which was spontaneously and completely aborted by day 34 of the third study cycle after taking mifepristone on day 14 (LH +2). This woman continued in the study and completed 6 treatment cycles.
Figure 3.4  This figure illustrates the download test data from the CPEFM used by the woman who missed the LH peak due to monitor error. The E3G and a subsequent low LH peak occur “too early” in the cycle hence the monitor does not declare peak fertility. The closed circles represent the LH signal, and the open squares represent the E3G signal. Fertility status is indicated as L = low fertility, H = high fertility, & P = peak fertility. The solid green line indicates the monitor declaring an LH peak because of cross-reactivity of rising $\beta$hCG with the LH assay.

The mean frequency of sexual intercourse was 1.8 episodes per week in 167 treatment cycles in which sexual activity was recorded. If we assume the probability of pregnancy in the treatment group is similar to the control group (0.25 - 0.32), the expected number of clinical pregnancies during the 178 cycles (in which 140 were exposure cycles)
studied should be between 35 – 48.3. The observed number was 2. Therefore, the efficacy of the method is 94.3% (95% confidence interval 75.4 – 98.7) - 95.9% (95% CI 82.5 - 99.0). When calculating the efficacy of the method, we excluded the 29 cycles during women were not exposed to a risk of pregnancy, and the three cycles in which mifepristone was taken in the follicular phase (Table 3.2).

### 3.3.3 Contraceptive efficacy of luteal phase administration of mifepristone

In 145 cycles in which mifepristone was taken in the early luteal phase (within 2 days of the urinary LH surge) 117 were exposure cycles. Exposure status was unknown in eight cycles and in 20 cycles women were not at risk of pregnancy. In the 117 exposure cycles, there was only one clinical pregnancy.

In 19 (10.7%) cycles, the monitor declared no LH surge but mifepristone was given as coitus had taken place during the fertile period of the cycle (calculated according to the usual cycle length and usual day of LH surge). Occurrence of ovulation was confirmed by serum progesterone of >5 nmol/L in all 19 cycles and treatment was administered prior to day 21 of the cycle in each case (between day 13 – 21 of the cycle (Mean 16.9 (SD +/-2.1) days). There were no pregnancies in these cycles.

If the probability of pregnancy in all exposure cycles in the study is 0.25 - 0.32 (the same as that of the control group), 34 - 46 clinical pregnancies would be expected in the 136 ovulatory cycles in which mifepristone was taken in the luteal phase. The observed number was one. Hence, the contraceptive efficacy of luteal phase mifepristone is between 97.1% (95% CI 78.00 – 99.6) and 97.8% (95% CI 83.9 – 99.7) (Table 3.2).

### 3.3.4 Performance of the home use hormone monitor

In 140 treatment cycles an LH surge was identified by the monitor, which equates to 90.9% LH surge detection when calculated for perfect use cycles; and 80.5% when
Table 3.2  Treatment cycle details.

<table>
<thead>
<tr>
<th>Mifepristone administered</th>
<th>Total number of cycles</th>
<th>Exposure cycles</th>
<th>Unknown exposure</th>
<th>No Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>167</td>
<td>136</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>• In follicular phase</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>• In luteal phase</td>
<td>164</td>
<td>136</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>Early luteal phase</td>
<td>145</td>
<td>117</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>LH +2</td>
<td>127</td>
<td>100</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>LH +1</td>
<td>17</td>
<td>16</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>LH+0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>In luteal phase (Unknown LH status)</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>• Mifepristone not given</td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

(LH surge missed, at risk of pregnancy but after day 21)

(Anovulatory cycle n = 1
LH surge missed & no risk of pregnancy n = 5)

Total 178 141 8 29
imperfect use cycles are also included in the total. In 127 cycles this was confirmed by a subsequent rise in serum progesterone of >5 nmol/L in the early luteal phase. This information was not available from 9 cycles (blood samples lost or not collected). In the remaining 4 cycles serum progesterone was between 2 – 5 nmol/L, one or two days following the urinary LH surge as detected by the monitor. This may have been due to an early detection of the first significant rise in urinary LH. None of these five cycles were prolonged after taking mifepristone hence; it is unlikely that they were anovulatory.

There was a total of 38 (21.3%) cycles in which an LH surge was not detected. Among them, one (0.6%) was an anovulatory cycle, defined by serum progesterone not rising above 5 nmol/L in the mid-luteal phase. In three (1.7%) other cycles we administered mifepristone on day 19, before the monitor had identified an LH surge. Serum levels of progesterone (taken on the day of administering mifepristone) confirmed that in these cycles mifepristone was administered in the follicular phase. All three cycles were prolonged (43 - 52 days).

In the remaining 34 cycles an LH surge probably occurred (as suggested by a rise in serum progesterone of >5 nmol/L) but was not identified by the monitor. Fourteen were missed due to monitor method failure (7.9%) and 20 were a consequence of imperfect use of the system (11.2%).

3.3.5 Cycle length
Mifepristone, when given in early luteal phase, did not significantly affect the cycle length (p = 0.35). The mean of the usual cycle length was 28.3 days (SD +/-1.3) and during the treatment cycles it was 28.0 days (SD +/-1.9).

3.3.6 Side effects
Women kept a record of vaginal bleeding in 139 out of the total 144 cycles where mifepristone was taken on LH +2. Mifepristone induced vaginal bleeding within 72
hours in 21 cycles (15%). In further 19 cycles, volunteers took mifepristone in the luteal phase but the LH status was not known. In 17 of those cycles (>89%), mifepristone induced a vaginal bleed.

Serum progesterone values in blood samples taken just prior to mifepristone administration were available for 136 cycles. The mean serum progesterone value was significantly (p <0.0001) higher in those cycles where mifepristone induced bleeding when compared to the mean value for the cycles without bleeding (21.72 (SD +/-9.04) nmol/L Vs. 13.33 (SD +/-6.23) nmol/L).

Two women spontaneously reported improvement of their pre-menstrual symptoms during cycles in which mifepristone was administered, while one reported worsening. In one woman hepatic alanine aminotransferase (ALT) was elevated at 103 U/L (normal range 10 – 40 U/L) at the end of the study but returned to normal within 2 months. One woman complained of diarrhoea 12 hours post mifepristone in one cycle, 3 reported menstrual cramping within 72 hours of taking mifepristone; 2 women reported a reduction in menstrual blood loss.

3.4 Discussion
A single dose of 200 mg of mifepristone administered once a month is an effective contraceptive method with an overall efficacy of 95% increasing to 97% if administered at the correct time (i.e., the early luteal phase). Thus, our results are in agreement with the findings of the previous study by Gemzell-Danielsson (Gemzell-Danielsson et al. 1993).

One criticism of previous work in this field has been the lack of a suitable control group for the subjects studied. Unlike the Gemzell-Danielsson study, we were able to compare the results with a contemporaneous control group using the same methodology in the same cultural setting. In this control group, if sexual intercourse took place on a fertile
day the probability of a pregnancy was 0.25 - 0.32. The calculated probability of pregnancy in a cohort of couples monitored during a study of natural family planning (WHO 1983) was 0.486 if intercourse took place 3 days prior to and a day after the peak day of mucus discharge. The difference in the probability of pregnancy between our study and a variety of other published series (Table 3.3) may be explained by the fact that we have extended our definition of the fertile period to 6 days (3 days prior to the urinary LH surge until 2 days after). Other authors (Wilcox et al. 1995) have shown that the likelihood of conceiving during an ovulatory cycle to be 0.37 (95% confidence interval 0.31 to 0.48) if daily sexual intercourse took place during a 6 day fertile period (four days before and a day after ovulation). The lower frequency of intercourse in our group (untimed intercourse averaging 1.7 per week) may also explain the lower probability of pregnancy.

Table 3.3 Probability of clinical pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Number of exposure cycles</th>
<th>Number of pregnancies</th>
<th>Probability of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilcoxon study+</td>
<td>129</td>
<td>34</td>
<td>0.26</td>
</tr>
<tr>
<td>Our control group+</td>
<td>37 - 48</td>
<td>12</td>
<td>0.25 - 0.32</td>
</tr>
<tr>
<td>Our treatment group+</td>
<td>140 - 151</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>(monitor + mifepristone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Our treatment group+</td>
<td>136 - 143</td>
<td>1</td>
<td>0.007</td>
</tr>
<tr>
<td>(mifepristone in luteal phase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-D study group*</td>
<td>124</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>WHO study*</td>
<td>72</td>
<td>35</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*The length of the fertile period defined as 4 days
+The length of the fertile period defined as 6 days
Mifepristone as a once-a-month pill

The limiting factor in this once-a-month approach to administering anti-progesterone is the accurate detection of the LH surge. Clearly, the failure to detect accurately the LH surge has a big impact on the overall effectiveness of the method. Using laboratory assay of LH in blood or urine to identify ovulation is neither practical nor convenient for long term use in the general population. The monitor provided us with an opportunity to overcome these problems. Gemzell-Danielsson reported 49% accuracy using home LH detection sticks. Although the monitor performed better (over 80.5% accuracy), both of these methods remain below the required standard. We studied 32 women over a total of 178 cycles. Imperfect use of the system accounted for failure to identify an LH surge in 11.8% cycles while 7.9% were due to monitor method failure. Compliance difficulties are associated with all contraceptives and non-compliance in approximately 12% of cycles is probably no worse than with any other method which demands action from the user, for example, compliance rates reported from oral contraceptive pill users range from 3.4% - 100% (Wheble et al. 1981, Hamilton et al. 1989, Molloy et al. 1985). Although our study population consisted of women who were motivated and committed and some of them already had experience in using natural family planning methods, they found the short, inflexible testing window set on day 1 of the cycle to be particularly demanding. This is inconsistent to couples using the monitor in order to get pregnant (Bonnar et al. 1999). The prevalence of imperfect use is likely to rise in the general population compared with that typical of a research study.

During the course of the study we developed an algorithm (Figure 3.1) for the administration mifepristone if an LH surge was not identified. In 19 exposure cycles (out of 28 cycles in which an LH surge was not identified) mifepristone was administered using this algorithm and there were no pregnancies. Given that the methods available to be used in real life to time the administration of mifepristone cannot be 100% accurate, such an algorithm will be essential to deal with a missed LH surge.
In our study, mistimed administration of mifepristone led to predictable effects. When administered during the proliferative phase of the menstrual cycle, mifepristone inhibited follicular development, and delayed the mid cycle LH surge, leading to a delay in ovulation and subsequent prolongation of the menstrual cycle (Liu et al. 1987, Luukkainen et al. 1988, Swahn et al. 1988). Ovulation may occur later in that cycle, leaving women at risk of conception. In our study, when administered in the late follicular phase (in error) in 3 cycles, mifepristone prolonged the cycle length (43 – 52 days). The women were advised to use condoms for the remainder of that cycle and none of the three cycles resulted in pregnancy.

Figure 1. Algorithm for administering mifepristone when the LH surge is not identified.

```
LH surge not identified by the monitor
a) within 3 days of the expected day of the surge
   or
b) by day 19 of the cycle

at risk of pregnancy  not at risk of pregnancy

administer mifepristone  no mifepristone

condoms for the rest of the cycle  condoms for the rest of the cycle
```

Administration of mifepristone in the mid or late luteal phase induces a bleed within a few days of treatment, which may or may not be followed by a second bleed at the time of expected menstruation (Shoupe et al. 1987; Swahn et al. 1988). The disturbance in vaginal bleeding induced by a method of contraception is an important side-effect because of its potential impact on acceptability. In our study, in 17 out of the 19 cycles...
where mifepristone was taken after ovulation (the LH status unavailable and probably later than on LH +2), inter-menstrual vaginal bleeding occurred (89.5%). Moreover, there was an increased risk of bleeding seen in those women who may have taken mifepristone slightly later in the LH +2 window. The mean serum progesterone concentration was significantly higher in those women who had bleeding after taking mifepristone within LH +2, when compared with those who did not. The higher serum progesterone value in some on LH +2, could be due to a delayed identification of the first significant rise in urinary LH, or because of a more rapid increase in serum progesterone due to early ovulation. Nevertheless, in our group of women, in all cycles where mifepristone induced a vaginal bleed, a second bleed occurred at the time of the expected menses. Therefore, while the bleeding may have been inconvenient, it did not jeopardise efficacy or continued use of the method. There was less inter-menstrual bleeding (15% of the cycles) reported in our study when mifepristone was taken within LH +2, less than half of that reported by Gemzell-Danielsson et al. (32%). This is possibly due to the fact that the majority of women in our study received mifepristone at the correct time. In their study, in 51% of the cycles, mifepristone was taken between 3 and 5 days after the LH surge. Anyhow, this predictability in the bleeding pattern following luteal phase administration is likely to be mere inconvenience, as opposed to the follicular phase administration.

The mean serum progesterone value was significantly more in those women who had mifepristone induced bleeding in the early luteal phase, when compared with those who did not. The higher serum progesterone value in some on LH +2, could be because of delayed identification of the first significant rise in urinary LH, or because of a faster increase in serum progesterone due to early ovulation.

The upset in menstrual pattern that may follow administration of mifepristone in the proliferative phase or in mid / late luteal phase can be unacceptable to women. However, there is new evidence (Mations et al. 2002; Brown, A. unpublished observation) that the
time window for administering once a-month mid-cycle mifepristone is wider than previously thought. This may be tested in the future where mifepristone is taken on a specific day of the cycle, arbitrarily chosen according to each woman’s usual menstrual cycle length.

Women are not exposed to the risk of contraceptive failure unless they have intercourse on a fertile day. Therefore, when calculating the contraceptive effectiveness of the method we excluded those cycles where the women reported that no intercourse had occurred during the fertile period and those cycles where insufficient information was available to determine whether intercourse has taken place. In our study, women marked only the days in which they had intercourse on the diary card, hence leaving no way to distinguish the days they forgot to mark sexual activity from those days when they actually did not have any. One might object that those who did not keep a record, still may have had intercourse during the fertile period and were nevertheless at risk of pregnancy. On the other hand, complex and more detailed diary cards may encourage errors of omission.

In conclusion, the use of the combination of home use fertility monitor with once a month administration of mifepristone (especially if mifepristone is administered at the early luteal phase) is an attractive contraceptive option with minimal side effects. However, to be an effective contraceptive method, the women have to be committed to using a device, which identifies the LH surge, in order that the pill can be taken at the correct time in the cycle. Whilst this regimen may be acceptable to motivated women, it may be regarded as too complicated for others to adopt on a routine basis. There was evidence of such non-compliance in this study, with 11.2% of LH surges being missed as a consequence of imperfect use of the monitor. Unfortunately, it is difficult to envisage how an easier way of defining the correct timing, which obligated less compliance, could be devised.
CHAPTER 4

SEX, LIES AND NON-COMPLIANCE

"Keep watch also on the faults of the patients, which often makes them lie about the taking of things prescribed"

Hippocrates.
4.1 Introduction

When the first woman on earth chose to contradict the instructions of her Provider in the Garden of Eden, expecting her descendants to comply perfectly with a contraceptive regimen may seem rather unrealistic. However, regardless the culture, race or the religious beliefs of a woman, unintended pregnancy is undoubtedly a major life-changing event. The consequences of such pregnancy generally make it unwelcome. Non-compliance with a particular contraceptive method is linked with an increased risk of unintended pregnancy (Rosenburgh et al. 1995). Therefore, conventionally it has been assumed that the users of contraception are ‘highly motivated’ because the consequence of non-compliance – pregnancy - is so obvious, and so significant. Studies involving organ transplant recipients have shown however, that no consequence of poor compliance is severe enough - not even the rejection of a transplanted kidney - for all patients to follow their prescribed regimen reliably (Rovelli et al. 1989). Converging evidence from all disciplines, in the treatment of chronic disease that requires suffers to adhere to particular medical regimens over a long period of time, indicates that poor compliance is pervasive (Haynes 1979). Furthermore, women on contraceptives are not suffering from a disease.

The studies that have evaluated the patient’s compliance with oral contraceptives show a wide range between the highest (100%) and the lowest (3.4%) compliance rates (Wheble et al. 1981, Molloy et al. 1985; Hamilton et al. 1989). This discrepancy may be due to the methodology of the studies as well as the differences in the patient characteristics - there is no “gold standard’ measurement for patient compliance (Spilker 1991).

Poor compliance – in both clinical practice and research – is associated with a number of factors. Though several patient characteristics (e.g. age, education, socio-economic background (Rapoff 1986; Meichenbaum & Turk 1987; Potter et al. 1991), characteristics of the treatment regimen (e.g. frequency of dosing, side effects (Puller 1988; Rapoff et al. 1989; Cromer et al. 1989)) and outcome characteristics (e.g. treatment of incurable or terminal illnesses (Spilker 1991)) have been associated with
non-adherent behaviour, there are no reliable and universally applicable predictors of non-compliance (Peck & King 1982; Luscher et al. 1985; Haynes 1986). Given the relatively crude measures of compliance that are available, caution should be taken when trying to correlate compliance with the outcome.

When a contraceptive method is used imperfectly, its effectiveness may differ from that during perfect use (Dominik et al. 1999). Clinical trials to determine the efficacy of new contraceptive regimens depend on full co-operation of the volunteers. Little is known about the prevalence of non-compliance in contraceptive trials. In these trials, volunteers are often required to keep daily records of events such as pill taking, sexual activity, vaginal bleeding and side effects in order to comply with a protocol. Contraceptive efficacy is usually compared with the estimated conception rates among non-users, which themselves directly rely on the accuracy of menstrual cycle data. Few women in real life regularly keep records of their menses or sexual activity hence, reporting errors are common. Moreover, incorrect reports of perfect compliance can under estimate use-effectiveness rates. Investigators generally depend on self-reporting (SR) of non-adherence behaviour to detect the prevalence of imperfect use.

We observed the behaviour in 32 women who took part in a trial assessing the efficacy of a novel method of contraception (Hapangama et al. 2001b). The women took 200 mg of mifepristone two days following the mid-cycle LH surge (measured in urine) as a once-a-month contraceptive pill. Mis-timed administration of mifepristone can disrupt the menstrual cycle, and can also leave the women at risk of conception. A home use fertility monitor that could store data on daily testing events was used to time the administration of mifepristone. While making minimal demands on the users, the method provided objective, long-term data on their routines. Since the investigator saw participants once each cycle throughout the study, we were able to compare the results of this method with the traditional self-reported incidence of compliance.
4.2 Subjects & Methods

4.2.1 Subjects
Data were collected during a study assessing the feasibility of administering mifepristone as a once a month contraceptive pill and detailed study methodology is reported elsewhere (Chapter 3; Hapangama et al. 2001b). Thirty-two sexually active women were enrolled from a large family planning clinic in Edinburgh. All subjects gave written informed consent to participation and the Lothian research ethics committee approved the study. Data was collected from a total of 178 cycles with each subject contributing between one and eight cycles. It was not possible to retrieve compliance data from the monitors in 28 of the study cycles, due to infrequent downloading of the monitor, and lost or broken devices. The study started on day 1 of the menstrual period following screening, and lasted for up to seven consecutive menstrual cycles in which subjects took a single tablet of 200 mg mifepristone once per month. The timing of mifepristone depended on detection of the LH surge, which in turn depended on compliance with daily urine testing.

4.2.2 Methods
All women underwent screening at the time of recruitment including routine physical and gynaecological examination. They were provided with a home use fertility monitor (Unipath, Bedford, UK). The system comprises a hand-held monitor and disposable dual-assay urine test sticks, and is used to detect simultaneously LH and E3G levels in early morning urine. The system will delineate three levels of fertility (Low, High and Peak Fertility) according to the optical signal changes detected. At the start of each menses, the subjects pressed the ‘m’ button on their monitor to initiate that cycle of use, at a time suitable for testing the first urine of the day. For the rest of the month, the subjects were required to consult the monitor display each morning (three hours either side of the time when ‘m’ button was set) to determine whether they needed to perform a test that day. Outwith this six hour time window the system would not accept a test. The monitor requests one test every day for up to a total of 10 or 20 tests, depending on the length of the woman’s cycle, and the timing of her LH surge. Embedded software within
the monitor collects, analyses and, stores data for several months (Figure 2.1; Figures 3.2 & 3.3).

The correct way to use the monitor (including the importance of the testing window) was demonstrated to all subjects at the time of recruitment and written information was given. Subjects were advised to contact the investigator immediately if they were not able to perform a test during a critical period. The investigator was available by telephone seven days a week.

Subjects were reviewed by the investigator each cycle, on day LH +2 when mifepristone was taken.

All subjects kept a menstrual record card, recording all vaginal bleeding experienced during the study and the days on which they had sexual intercourse. They also marked the day of the LH surge as identified by the device and the day of taking the study medication.

**The following definitions were created for the purpose of the study.**

The period of time between the calculated earliest day an LH peak is likely to occur and the day of the actual LH surge in each cycle was defined as the **Critical period** for each patient. **The Fertile period** of the cycle was defined as three days before until two days after the urinary LH surge (LH -3 to LH +2). **Exposure cycles** were cycles in which women reported having sexual intercourse at least once during the fertile period.

**4.2.3 Statistical Methods**

Some of the compliance data were summarised for descriptive purposes using numbers of cycles or tests as denominators. However, statistical inference was carried out on data aggregated to patient level, in order to take account of possible heterogeneity in behaviour amongst patients, which might have invalidated analyses using data from individual cycles or tests. Thus percentages of tests missed were calculated for each
patient, and these were tested for association with demographic data using Pearson correlation. Similarly, percentages of tests missed at different stages of the cycle, and when on and off the treatment regimen, were compared by paired t-tests. A two-sample t-test was also used to compare the percentage of tests missed in patients who dropped out and those who did not.

### 4.3 Results
Table 4.1 shows the demographic characteristics of the women who took part in the study.

**Table 4.1 Demographic data**

<table>
<thead>
<tr>
<th>Demographic feature</th>
<th>N = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>18 – 39</td>
</tr>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>30 (+/- 5.4)</td>
</tr>
<tr>
<td>BMI</td>
<td>19 – 38</td>
</tr>
<tr>
<td></td>
<td>Mean (+/- SD) 23.6 (+/- 4.3)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7 (21.9)</td>
</tr>
<tr>
<td>Non-smokers (%)</td>
<td>21 (65.6)</td>
</tr>
<tr>
<td>Ex-smokers (%)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>Previous pregnancies 1+ (%)</td>
<td>19 (59.4)</td>
</tr>
<tr>
<td>Never pregnant (%)</td>
<td>13 (40.6)</td>
</tr>
<tr>
<td>Ever abortion (%)</td>
<td>15 (46.9)</td>
</tr>
<tr>
<td>Married / Co-habiting (%)</td>
<td>28 (87.5)</td>
</tr>
<tr>
<td>Single (with a regular boy friend) (%)</td>
<td>4 (12.5)</td>
</tr>
</tbody>
</table>

162 cycles were analysed, out of which 150 were study cycles. Data were collected during an additional 12 cycles in which subjects were using a barrier method throughout the cycle and mifepristone was not given.
In total 2013 tests were requested by the monitor during the 162 cycles analysed (12.4 tests per cycle, 95% C.I. 11.8 - 13.0), and 494 were missed (24.2% (95% C.I. 16.5 - 31.5). On average three tests (95% C.I. 2.4 - 3.6) per cycle were missed.

4.3.1 Compliance during the intention to treat cycles
During the 150 cycles, in which women were relying on the study method as their only contraception, a total of 1816 tests was requested by the monitor and 411 tests were missed (22.6% (95% C.I. 15.2 - 30.1).

Compliance before and after identifying the peak
In the days before the women knew an LH surge had occurred, 23% of the requested tests were missed (95% C.I. 16 - 30)) (260 out of a total 1160). Women were not more likely to miss tests before the surge than after it (t = 0.57, NS).

Concordance of monitor data with the self-reported data
In 68 cycles (42%) women failed to test at all on a day of the cycle, which was critical to the accurate detection of an LH surge. In 27 of these cycles the monitor did not detect an LH peak. In the remaining 41 cycles despite critical tests being missed the monitor did detect an LH peak. Women admitted to not performing tests in 24 cycles (14.8 %). In the other 44 cycles women did not report missed tests and only admitted to it once the investigator showed the downloaded monitor data to them. Some women actively fabricated the information, which they reported to the investigator. In one of these cycles when contacted, a woman declared detecting an LH peak on a day (day 13 of the cycle) when she had not performed a test at all (Figure 4.1). Another woman performed the tests correctly and identified an LH peak on day 13 but forgot to inform the investigator and failed to obtain the mifepristone tablet as per protocol, when contacted on day 19 of the same cycle, she claimed that the monitor had not detected an LH peak.
Figure 4.1 This figure illustrates the monitor display as seen by the volunteer each day

Empty box = low fertility, Half filled box = high fertility. Underneath each box, a solid line represents a correctly performed test while a missed / inaccurately performed test is represented by a broken line. Volunteer using this particular monitor neither performed a test, nor did the monitor show peak fertility on cycle day 13. However, she declared detecting peak fertility on day 13 (see Figure 3.2 for perfect compliance).

Compliance during the critical days
Compliance with urine testing as monitored by the system was significantly higher during the critical period (15.6% (95% C.I. 9.5 - 21.7) when compared to the non-critical days (27.5% (95% C.I. 17.9 - 37) (paired t-test, t = 3.64, p <0.01). The self reported percentage of missed tests during the critical period was 2.7% (95% C.I. 1.0 - 4.3) which was significantly lower than that detected by the monitor (t = 4.48, p <0.001).

4.3.2 Compliance during the study suspension interval
In six out of these 12 cycles, the monitor was not able to identify an LH peak due to imperfect use. During this period, a significantly high percentage of tests (41.2 % (95% C.I. 22.4 – 60.0)) were missed when compared to the study cycles (22.6% (95% C.I. 15.2 - 30.1) (t = 2.9, p = 0.015).

4.3.3 Compliance and sexual activity
There was no correlation between the frequency of sexual intercourse per cycle and the number of missed tests per cycle (correlation coefficient was +0.01). The percentage of missed tests were compared between the exposure and non-exposure cycles in 12 women who had at least one exposed and one unexposed cycle. There was no significant difference (paired t-test, t = 0.06, NS, 95% C.I. for differences between exposed and unexposed –8.3 to +7.8) in compliance during exposed and non-exposed cycles.
4.3.4 Demographic features and compliance

Age was negatively correlated with the percentage of missed tests – younger women missed more tests ($r = -0.36$, $p < 0.05$). There was no apparent relationship between compliance with the method and the number of pregnancies, number of previous abortions, or number of living children (Table 4.2). All women included in the study had some form of tertiary education and were either employed or were undergoing tertiary education during the study period.

Table 4.2 Demographic data and compliance

<table>
<thead>
<tr>
<th>Demographic feature</th>
<th>Number</th>
<th>Mean % non-compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
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<tr>
<td>18–24</td>
<td>5</td>
<td>36.8</td>
</tr>
<tr>
<td>25–29</td>
<td>11</td>
<td>24.2</td>
</tr>
<tr>
<td>30–34</td>
<td>7</td>
<td>16.8</td>
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<tr>
<td>35–39</td>
<td>8</td>
<td>15.2</td>
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<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19–20</td>
<td>6</td>
<td>13.7</td>
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<td>21–25</td>
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<td>27.9</td>
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<tr>
<td>26–38</td>
<td>7</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>7</td>
<td>24.3</td>
</tr>
<tr>
<td>Never</td>
<td>19</td>
<td>21.7</td>
</tr>
<tr>
<td>Ex</td>
<td>4</td>
<td>18.4</td>
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<tr>
<td><strong>Previous pregnancies 1+</strong></td>
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<tr>
<td>Never</td>
<td>14</td>
<td>21.9</td>
</tr>
<tr>
<td><strong>Ever abortion</strong></td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>25.3</td>
</tr>
<tr>
<td>No</td>
<td>17</td>
<td>21.9</td>
</tr>
<tr>
<td><strong>Married/ Co-habitng</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td><strong>Single</strong></td>
<td>3</td>
<td>18.1</td>
</tr>
</tbody>
</table>
4.3.5 Compliance among dropouts
We analysed cycle data from the seven women who discontinued the study before completion. They missed a significantly higher percentage of tests (44.4%) when compared with the other 25 women who completed the study (17.4%) (95% C.I. of the differences between the two percentages 10.6 – 43.3) (t = 3.39, p = 0.002). The dropouts missed tests on a critical day in 16 out of the 22 cycles although admitted to missing tests in only four. Therefore, the self reported incidence of non-compliance during the critical period was 18.1%, while the monitor detected incidence was 72.7%.

4.4 Discussion
The home use fertility monitor offered us an important methodological advance in providing reliable data on the incidence and the magnitude of non-compliance with the contraceptive method studied, and information on when exactly the imperfect use occurred. We sought to achieve significantly high compliance rates with employing this monitoring system in a contraceptive regimen, which appeared to be acceptable to women (Rimmer et al. 1993; Glasier et al. 1999). Women were counselled at the start of the study regarding the importance of performing urine tests accurately in order to identify the time to administer mifepristone. They were aware that if mifepristone was not taken, they would be at risk of pregnancy. Since the teratogenic effects of mifepristone is not known women considering participation in the study were advised that if pregnancy occurred they should consider termination. Despite this, the information collected from the monitor demonstrates that women failed to perform 24.2% of the tests in the 162 cycles analysed. Moreover, in 42% of the cycles women missed tests during a day that an LH surge was likely to have occurred, i.e. at an absolutely vital time for contraceptive efficacy.

Although non-compliance is common, by inviting only a selected group of women (all with tertiary education) who appeared to be motivated and committed we sought to exclude those who might be poor compliers. We expected the positive health benefit, i.e. avoidance of pregnancy from our ‘new’ treatment may become their motivation to
comply with the protocol. Interestingly, the medical profession is reportedly poor in identifying non-compliers (Gilbert et al. 1980). Users of our method were reasonably young (mean age 30 years) and were healthy. The serious outcome of non-compliance (pregnancy) was rare. Women may have found daily requirement for urine testing using the monitor on a long-term basis to be complicated or inconvenient. Side effects of the method (although fewer than in most other methods available) may not have been acceptable to them or they may not have had faith in the effectiveness of the method although this seems unlikely since they did not revert to condoms. Considering these odds, it is not difficult to infer why compliance problems were frequent.

For a volunteer, taking part in a research study usually involves extra effort. Women in our study were required to attend clinics, answer questions honestly, test samples accurately as requested (daily urine), allow required tests to be performed (serum progesterone assay), obtain medicines prescribed and keep accurate records of events (sexual intercourse, menstrual bleeding etc) in order to comply with the study protocol. Common sense suggests that with increasing experience the women may trivialise the need for testing on days in which an LH surge was unlikely to have occurred. All women were made aware of the critical period for testing urine at the start of the study and as expected, women missed fewer tests during this period. Incomplete understanding of how to be compliant with a regimen has been suggested as a common reason for poor compliance (Rosenberg et al. 1995). Women in our study were given detailed verbal and written information on using the monitor at the start of the study. The importance of accurate use of the device according to manufactures guidelines was stressed at each monthly visit. Since there was no significant difference in the percentage of missed tests between the first study cycle and the last study cycle, it is difficult to support the suggestion that in the earlier cycles women were less confident about how to use the method or more confident in their ability to guess the correct time for testing (Lusher et al. 1985).
There was a highly significant difference between the patient self-reported percentage of missed tests (2.6 %) and that detected by the monitor (15.6 %) during the critical period (Figure 4.2 & 4.3). This discrepancy was even greater among the seven women who discontinued the study before completion, and is consistent with previous studies that reported significant overestimation of compliant behaviour with SR (Potter et al. 1996). In 1996, Potter and colleagues studied SR incidence of missed pills with the data from an electronic device with diary entries among a group of OC users, and the compliance ranged from 25% (Electronic Device data) to 66% SR. Patients tend to tell the doctors what they think the doctor wishes to hear. If they assume that doctors perceive patient non-adherence as a judgmental disappointment, they may feel guilty to report non-compliance.

Although, older women tended to miss fewer tests, the other demographic characteristics such as parity, previous abortions, number of living children and educational background did not correlate with the number of missed tests. Others have also reported a positive correlation of increasing age with adherence behaviour (Waterhouse et al. 1993). Neither did frequency of sexual activity or exposure to the risk of pregnancy correlate with the missed tests. However, since the information about sexual activity was only collected by self-reporting, this association should be accepted with caution.

Most investigators work on the assumption that a patient who complies with one aspect of a clinical protocol (e.g. attending the clinic as directed) also adheres to all other aspects of the study, though this might not always be the case. The monitor supplied data on daily testing of urine, which was only one part of the contraceptive method. We cannot infer from those data, how well the women in our study complied with the rest of the protocol, such as recording daily events (sexual intercourse, vaginal bleeding). With no means of testing this, for validity of our results we have to depend on our volunteers being truthful. Therefore, in future we have no other option but to work towards forming a true therapeutic alliance with our volunteers; and to come to an agreement with our patients rather than to impose a prescription or a protocol upon them.
In conclusion, the use of microelectronics monitoring systems such as the home use fertility monitor may improve our understanding of the extent of the problem of patient non-compliance, providing precise objective information that no other monitoring technique can produce. This understanding will empower us as health care providers, to adopt a no fault approach to behaviour relating to non-compliance and establish “a tailored consensual regimen” with the user which they are able to adhere to (Fink, 1976). This provides the opportunity to make the optimum use of potentially effective treatments and legitimate research evidence. Perhaps a small price to pay for such a return!
**Figure 4.2** Information collected from the monitor and the diary card from a 31-day cycle in which, the LH surge was identified. Mifepristone was administered on day 20 (LH +2)

**Panel I: Information downloaded from the monitor**
Level of fertility displayed to the woman on each day of the cycle.
- Low fertility
- High fertility
- Peak fertility

Data on testing events.
- Test performed
- Test missed

**Panel II: Data recorded on the diary card by the woman (Self-report)**
B = days of vaginal bleeding  
X = days in which sexual intercourse had occurred  
✓ = tests done during the critical period  
T = day when mifepristone was taken

**Panel III: Downloaded information on signal levels of Luteinizing Hormone (LH) and Oestrone-3-glucuronide (E3G) levels.**  
LH (●)  
E3G (□)  
M = Missed tests

**Note that on several days – the woman reported performing (✓) the test when the monitor showed that she did not (M).**
Figure 4.3 Information collected from the monitor and the diary card from a 35-day cycle. As a consequence of missed tests the monitor did not identify the LH surge and, mifepristone was not administered.

**Panel I: Information downloaded from the monitor.**
Level of fertility displayed to the woman on each day of the cycle.

- Low fertility
- High fertility

Data on testing events
Test performed | Test missed

**Panel II: Data recorded on the diary card by the woman (Self-report)**
B = days of vaginal bleeding  √ tests done during the critical period
X = days in which sexual intercourse had occurred

**Panel III: Downloaded information on signal levels of Luteinizing Hormone (LH) and Oestrone-3-glucuronide (E3G) levels**
LH (●) E3G (□)  M = missed tests
CHAPTER 5

THE ROLE OF A PERSONAL HORMONE-ASSAYING SYSTEM IN CLINICAL RESEARCH
5.1 Introduction

If sexual intercourse takes place on a fertile day of a woman's menstrual cycle, she may fall pregnant. The potentially fertile period in the cycle is determined by the time of ovulation. The duration of this fertile period however, is dependent upon the survival time of the sperm and the egg in the female genital tract.

The mid-cycle peak in the urinary levels of LH precedes ovulation by approximately 24 hours (Collins et al. 1996). A variety of self-detection kits or devices measuring urinary concentrations of LH has been used to determine the day of ovulation. The maximum survival time of the ovum is calculated to be less than 48 hours (Royston 1982). The rapid drop observed in the probability of conception after ovulation further supports a short life span for ova (Wilcox et al. 1995). The enlarging dominant follicle produces increasing levels of oestradiol in the late follicular phase (Kerin et al. 1981). Corresponding levels of E3G, the main metabolite of oestradiol - can be detected in the urine and have been used in conjunction with urinary LH to identify the potentially fertile period prior to follicular rupture and ovulation (Aldercreutz et al. 1982, WHO 1983, Schiphorst et al. 1985). Estimates using clinical data have suggested the number of fertile days in each cycle to be around 6 days, ending on the probable day of ovulation (Wilcox et al. 1995).

An EC can be effective by inhibiting or disrupting ovulation / fertilisation or interfering with the transport of the blastocyst, or inhibiting its implantation in the endometrium. Sex steroids have several effects on the ovulatory cycle and on the endometrium depending on the time of administration hence have been used as EC. Detailed understanding of the anti-fertility mechanism of these agents may help to select the most effective option for each individual depending on the time in the cycle when intercourse had occurred. Therefore, a reliable yet simple method of detecting the fertile period and the day of the ovulation is invaluable for research into EC.
The frequent and regular laboratory assays of steroid hormones in either blood or in urine have traditionally played a major role (in detecting the fertile period of the cycle, the cyclical hormonal changes, and the timing of ovulation) in research into female contraception. This approach is not only inconvenient for the volunteers but it is also expensive and time consuming to collect samples for daily assays. For example, usually the daily urine samples are collected and stored over several cycles and then analysed for hormone levels. Poor compliance with collecting specimens or wrongly labelled specimens may be difficult to detect, but if undetected can present difficulties in the interpretation of results. We analysed the practical aspects of using a microelectronic personal fertility monitor as an alternative to the laboratory based hormone assays, in a study that investigated the mechanism of action of an EC.

5.2 Subjects and methods

This was a prospective, randomised, double blind, crossover study undertaken in one centre. Twelve healthy women (mean age 33.3 (range 26 - 41 years)) with regular cycles (mean 27.6 days (range 25 - 30 days) and mean BMI 25.7 (range 20 - 34)) were recruited. They were all using a reliable non-hormonal method of contraception or were abstinent during the study. All subjects gave written informed consent to participation in the study, which was approved by the Lothian Research Ethics Committee.

A method was sought to provide a convenient means of identifying the fertile period prior to ovulation, and thereby time the administration of LNG. A home-use Fertility Monitor was used for this purpose. This product is similar to the Personal System of Contraception (Persona, Unipath Bedford UK) (Bonnar et al. 2000) in that it monitors E3G and LH in urine, but differs in that it has been specifically designed for maximising the chance of conception. Unipath Diagnostics Co., Princeton, NJ, markets the fertility monitor in the US and Unipath Ltd., Bedford, UK markets it in Britain as CPEFM (Figure 2.1).
This inter-active system comprises a hand-held monitor and disposable dual-assay urine test sticks, and is used to simultaneously detect LH and E3G levels in early morning urine. The monitor optically measures the intensity of the lines that form on the test sticks after sampling, and the system will delineate three levels of fertility (Low, High and Peak fertility) according to the optical signal changes detected. Low Fertility will be displayed from day 1 of the cycle, until the hormone levels rise above the baseline levels. A change from Low to High Fertility is triggered by detection of elevated E3G levels. The change from High to Peak Fertility is triggered by the detection of an LH surge. Peak fertility is displayed on the day of the LH surge and on the following day (Figure 3.2).

Each woman was studied during four cycles, and was issued with a monitor at the beginning of the study. Subjects were asked to use the CPEFM according to instructions, and familiarised themselves with the monitors by using it during a pre-study cycle to identify the days of high fertility and the day of the LH surge. They also recorded days of vaginal bleeding.

Data from the pre-study cycle was used to predict the timing of the LH surge and of the fertile phase during the study cycles and thereby to predict when treatment should be administered. The three study cycles followed immediately after the pre-study cycle. Six subjects were randomly assigned to treatment arm A and received LNG in the first study cycle and placebo in the third study cycle. The remaining six subjects were randomised to treatment arm B and received placebo in the first study cycle and LNG in the third study cycle. The second study cycle was a “washout” phase for all women during which time they also received placebo tablets (Figure 2.2).

The randomisation list was produced using SPSS Rv.Bernouilli function such that each study number was randomly assigned to either treatment arm A or treatment arm B with the same probability i.e. 0.5.
Each subject collected a sample of early morning urine daily from the first day of the first study cycle until and including the first day of the menstrual bleed signaling the end of the last study cycle. Samples were frozen and later assayed in batches (with all samples from one subject assayed in a single batch) for measurement of urinary LH, E3G and P3G.

Quantitative assessment of urinary LH was performed using a LH MAIAclone kit (BIOSTAT-DIAGNOSTICS, Stockport, Cheshire, UK). This method incorporates two high affinity monoclonal antibodies into an immunoradiometric assay system and offers a working range of 1.5 – 200 mIU/ml. Urinary P3G was measured using a direct enzyme immunoassay (working range 0.16 µg/L – 20 µg/L), while a direct immunoassay was used to measure E3G levels (working range 8.36 nmol/L - 2140 nmol/L). Intra-assay and inter-assay coefficients of variation were 6% and 10% for E3G, 10% and 12% for pregnanediol and 3% and 5% for LH respectively (Yong et al. 1992). Only the intra assay variability was relevant since the specimens for a given woman were analysed on the same assay. Geometric means of daily replicates were divided by the respective daily creatinine concentration to correct for variations in the dilution of the urine specimen.

During study cycles 1 and 2 women were asked to take the study medication on the first day of high fertility as identified by the monitor. However, by the third study cycle, the variation in the number of High Fertile days (range 0 - 9 days) meant that the monitor could not be used to administer medication in the fertile period in every cycle. Therefore, we had to adopt a different method of calculating the anticipated day of the LH peak for each cycle based on the monitor information from the previous cycles (including the pre-study cycle). Hence, in the third study cycle, the medication was taken two days prior to the anticipated day of the LH peak. In all cycles the first tablet was taken at 11.00 hours and the second at 23.00 hours. A sample of venous blood was collected 5 – 7 days after treatment, stored and later assayed for progesterone using Coat-A-Cont solid-phase
radio-immunoassay. The subjects kept a daily record of all vaginal bleeding experienced during the 4 cycles, the fertility status information displayed each day on the monitor LCD and the days on which the study medications were taken.

It was not possible to retrieve monitor data in three study-cycles due to lost or broken devices, and there were no daily urine samples available from an additional cycle as the woman was abroad on holiday. Therefore, a total of 32 study cycles were analysed.

For the purpose of the study the following definitions were created.

Definitions based on the quantitative data.

**A significant delay in the onset of next menses:** Delay of 5 or more days from the expected onset of menses (based on the mean cycle length for the 2 placebo cycles).

**The LH peak** was defined as a significant rise in urinary LH concentration, with a minimum of 50% rise above the average baseline level for 3 preceding days and which remained elevated for a minimum of 3 days.

**The first day of the LH peak** was defined as the day of the first significant rise (>50% above the baseline) seen at the beginning of the LH peak.

**Retrospectively predicted first day of the LH peak** for the treatment cycles was the calculated mean of the first day of LH peak in the two placebo cycles.

**A significant delay in the first day of the LH peak:** Delay of 5 or more days from the expected first day of the LH peak in the treatment cycle (based on the mean first day of the LH peak during the 2 placebo cycles).

**Luteal phase:** time from the day after the first day of the urinary LH peak (LH +1) until, and including, the day before the first day of the next menses.

**Follicular phase:** time from the first day of the menses until the day of the first significant rise in urinary LH (LH +0) inclusive.

Definitions based on the monitor data. **High fertile days:** days preceding the urinary LH peak as indicated by the monitor to be potentially fertile. The monitor was
programmed to display an additional day of high fertility following the 2 peak fertile days.

**Peak fertile days:** The day of the first significant rise in urinary LH (>50% above the basal level over the preceding 3 days) detected by the monitor. The monitor was programmed to display a further day of peak fertility following the first rise.

### 4.2.3 Statistical Methods

The compliance data was summarised for descriptive purposes using numbers of cycles or tests as denominators.

### 5.2 Results

Data from the monitor and from RIA of daily urines was available in 32 study cycles, and in 11 of these (i.e. one treatment cycles per volunteer) women took LNG on a High fertility day. In the remaining 21 study cycles, placebo tablets were taken on a similar day. In four out of the 11 treatment cycles, LNG treatment aborted and or significantly delayed the subsequent LH surge. However, in the remaining seven treatment cycles, LNG did not alter the day of the LH surge or the cycle length significantly. In addition, one of the placebo cycles was prolonged due to a spontaneous delay in the LH surge. Therefore, the 5 cycles, in which the LH peak was significantly delayed in comparison to the usual pattern were analysed separately (Group 2) to the other 27 cycles (which consisted Group 1).

#### 5.3.1 Group 1

**Results from laboratory reference method for urinary hormones**

The mean E3G levels in urine increased to maximum levels of 24.74 μmol/mol creatinine (95% C.I. 17.72 – 31.76), 1.2 days before the peak urinary LH value in the 27 ovulatory cycles. In these cycles, the mean concentration of peak urinary LH was 3.91 U/mol creatinine (95% C.I. 2.8 – 5.02) (Figure 5.1). As expected, the mean urinary P3G levels started to rise on LH +1.
The monitor warning of the LH surge

The days of high fertility recognised by the monitor was regarded as the warning of the LH surge, and subsequent ovulation. It had been reported that the increase in E3G production from the enlarging follicle, with a parallel increase in serum levels and the corresponding urinary levels were associated with the monitor indicating increasing incidence of High fertility status on the days prior to ovulation (Behre et al. 2000). Among the 11 women, the monitor provided on average 3.4 days (95% C.I. 2.6 – 4.2; range 0 – 9 days) of warning prior to the first day of the urinary LH surge. In 74.07% of the cycles the monitor provided between 1 - 4 days of warning prior to the first day of the urinary LH surge (Figure 5.2).
Comparison between the monitor peak fertility, and the urinary LH surge

The monitor detected an LH surge in 21 out of the 27 cycles in group 1. In all 21 cycles the monitor detected an LH surge within two days of the first day of the urinary LH surge measured by laboratory RIA (table 5.1, Figure 5.3). In these 21 cycles, the percentage rise in urinary (laboratory RIA) LH concentration above the baseline level (baseline value = average value of LH concentration during the preceding 3 days) was compared between the actual (laboratory RIA) LH peak day and the monitor peak day (Table 5.2). Except for 1 cycle, the daily urine samples analysed in the laboratory confirmed the LH percentage rise of > 50% above the baseline value on the day that the monitor declared an LH peak. In the remaining six cycles, an LH surge had occurred, but was not detected due to missed tests in one cycle; monitor error in two cycles; and could have been due to either of the above mentioned causes in three further cycles (no compliance data available for these three cycles).
Table 5.1  First day of the urinary LH surge (LH + 0) measured in the laboratory by RIA, relative to the first day of peak fertility indicated by the CPEFM monitor in group 1

<table>
<thead>
<tr>
<th>Day of the LH surge by laboratory RIA of the daily urine samples relative to the monitor peak</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before the first day of monitor peak fertility</td>
<td>3</td>
<td>14.3</td>
</tr>
<tr>
<td>On the same day as the first day of monitor peak fertility</td>
<td>16</td>
<td>76.2</td>
</tr>
<tr>
<td>After the first day of monitor peak fertility</td>
<td>1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Figure 5.3  Urinary LH and percentage of peak fertility status

- Percentage of peak fertility status
- Urinary LH

-15 -14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4
day relative to the first day of the urinary LH surge
Table 5.2  The average percentage rise of the LH above the baseline concentration in collected daily urine (laboratory RIA) in group 1

<table>
<thead>
<tr>
<th>The actual (laboratory RIA) LH peak</th>
<th>The average % rise from the baseline on the day of the monitor peak</th>
<th>The average % rise from the baseline on the actual (lab-based urinary) LH surge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before the first day of monitor peak</td>
<td>226 (SD +/-108.0)</td>
<td>244.1 (SD +/-196.5)</td>
</tr>
<tr>
<td>fertility (n=3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>On the same day as the first day of</td>
<td>357.2 (SD +/-264.2)</td>
<td>357.2 (SD +/-264.2)</td>
</tr>
<tr>
<td>monitor peak</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fertility (n=16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After the first day of monitor peak</td>
<td>13.64</td>
<td>105.13</td>
</tr>
<tr>
<td>fertility (n=1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3.2  Group 2

In these five cycles, the occurrence of a mid-cycle LH surge and subsequent ovulation, were either prevented or delayed (reflected by the absence of normal rise in urinary pregnanediol during the luteal phase). Nevertheless, a second E3G rise, a successive peak in urinary LH and a consequent rise in mid-luteal pregnanediol were observed later on, in the same cycle (Figure 5.4).

During these five cycles, at the expected time of ovulation for each woman the mean urinary LH reached peak concentrations of 1.1 U/mol creatinine (significantly lower than the value during the control cycles, p <0.0001). The urinary E3G also increased to a peak on the same day (17.48 µmol/mol creatinine) but thereafter decreased down to an early follicular level. Furthermore, these lower than usual LH peaks were not followed by an increase in the urinary P3G. Subsequently, a second urinary LH peak (3.1 U/mol
creatinine, SD +/-1.0) occurred 17.4 days later in that same cycle (Figure 5.4), and was preceded by a peak in the mean urinary E3G level (19.06 µmol/mol creatinine SD +/-4.14).

![Graph](image)

**Figure 5.4**  *Treatment cycle in subject S106.*

Note the delayed true LH peak on day 23 which is followed by an increase in the urinary pregnanediol levels. The white arrow represents the LNG administration.

**Comparison between the monitor peak fertility, and the urinary LH surge**

In three of these cycles, the monitor indicated peak fertility at the expected time for the respective volunteer (false positive). The monitor failed to identify every one of the second LH surges that occurred later in the cycles (between days 25 – 40). The reasons are as follows; In three cycles by the time the second LH surge had occurred, the monitor had ceased requesting tests; in one cycle the woman failed to perform a test requested by the monitor on the first day of the second LH surge (user error); in another cycle,
although a test was performed on the first day of the second surge, the monitor failed to identify it (monitor error) (Table 5.3).

<table>
<thead>
<tr>
<th>Reasons for the monitor not detecting an LH surge</th>
<th>User Error (%)</th>
<th>Monitor error (%)</th>
<th>Unknown n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Percentage of the total number of cycles</td>
<td>3.7%</td>
<td>7.4%</td>
<td>11.1%</td>
</tr>
</tbody>
</table>

5.3.3 Objective measurement of compliance with urine testing

Compliance data on daily urine testing was available in 26 cycles, and this information could not be retrieved from the monitors in 6 cycles due to infrequent downloading. The eleven women failed to perform 18.9 % (95% C.I. 16.7 – 25.1) of the total tests requested by the monitor. On average a woman missed between 2 – 3 tests of the average 12.2 tests requested (range 10 – 20 tests).

5.4 Discussion

A reliable and simple means to predict the potentially fertile period will provide essential information that can be used to influence the probability of conception. If the users can be reliably warned of the potentially fertile period in each cycle, that information can be employed in clinical practice, to time intercourse in order to avoid or permit the occurrence of a conception as desired. Such methods will assist in contraceptive research to monitor the frequently changing hormone levels, and to identify the fertile period.

Hormonal methods of EC are widely used to prevent pregnancy after unprotected coitus (Glasier 1997 review). Sex steroids that are used as ECs have several effects on both the ovulatory cycle and on the endometrium. These effects are dependent on the time in the
cycle that the EC is administered. Therefore, the accurate prediction of the potentially fertile period should enable investigators to identify the particular mechanism of the sex steroid that prevents conception when intercourse has already taken place.

The precision of the method that identifies the fertile period directly affects the accuracy of the estimated probability of conception following an act of unprotected intercourse. Therefore, one can see that the accuracy of the estimated efficacy of any EC method is directly dependent on the technique used to identify the fertile period.

Previous studies have confirmed that the home-use fertility monitor data correlated well with the laboratory measured urine hormone levels (Bonnar et al. 1999; Behre et al. 2000). In contrast to the laboratory assays that are inconvenient to women (e.g. collection of a large number of urine samples), time consuming, and expensive, the monitor was rather easy to use and was possibly less expensive.

The consensus of opinion is that the fertile period of the woman’s cycle is around six days, ending on the estimated day of ovulation (Wilcox et al. 1995). A peak in urinary LH levels preceded ovulation by about 24 hours (Collins et al. 1996). In our study, to test the hypothesis that LNG acts as a post-coital agent by abolishing the pre-ovulatory LH surge and by delaying ovulation, we wanted to administer LNG in the fertile period immediately before the mid-cycle LH surge. Therefore, we required in particular, the prediction of the impending LH surge to be accurate (the warning had to be at least one day, but not more than four days before the urinary LH surge), to administer LNG at a matching time in each cycle in the fertile period, so the observed effects could be compared. The correlation between the High and Peak fertility days as indicated by the monitor and the urinary LH surge, showed (Figure 5.2) that in 25.93% of the cycles in group 1, there was either no warning or the warning started at more than four days before the LH peak had occurred. When the treatment had to be administered on the actual first day of the LH peak, it created a problem when comparing the effects of LNG taken on or
before the day of the urinary peak. It is possible that the timing of LNG in the former group was "too late" to influence an event already well underway. Similar obstacles were encountered in data analysis when LNG was administered long before ovulation had occurred (as a result of the advance warning of the imminent occurrence of an LH surge more than four days before the actual surge). The Clearplan Easy device is designed for a different purpose (for couples intending conception therefore the users should continue to have intercourse until peak fertility is recorded) hence "the prolonged fertile period does not matter" (Bhiwandiwalla et al. 2001) is not a valid argument for the following reasons. The purpose of using the device is to time intercourse since not all couples are able to have intercourse every day – indeed, if they were, there would be no need to monitor ovulation. If users exhaust themselves by having intercourse for several days before the days of peak fertility, they may be less likely to have intercourse at a time when the chance of pregnancy is maximal.

We have previously reported a monitor error of 7.9% in identifying an LH surge (Chapter 3; Hapangama et al. 2001b), and a similar performance with about 92% accuracy in LH detection was expected in this group of patients. Therefore, non-compliance with urine testing was likely to be the cause for missing the LH surge in the four out of the six cycles in group 1. Women missing about 19% of the urine tests requested by the monitor is obviously significant and comparable to previous observations by our group (Chapter 4; Hapangama et al. 2001c). The monitor stored data simultaneously on the hormone signal levels and on the testing events (volunteer compliance) for several months and this information could be later retrieved. Among urine samples that have been collected daily over a period, a wrongly labeled one will be impossible to detect, yet may affect the results. In addition, self-LH detection kits have been commonly used to identify the LH surge, for example to time the administration of a particular agent, and to sample the endometrium at a particular time (Cameron et al. 1996). Obviously, inaccuracy in detecting the LH surge (or undetected non-compliance) in such studies directly affects and invalidates the results. Therefore, the monitor offered
us an important methodological advance in providing reliable data on the incidence of non-compliance with urine testing, and exactly when a test was missed.

Several possible improvements could be made to the system to achieve its full potential use in clinical research studies. Although the urinary LH surge detection based on a significant rise preceded by a similar rise in E3G may be adequate for most of the normal cycles, it will erroneously detect an LH peak that had been aborted. If the qualifying criteria included the rise in LH to remain elevated for a minimum of 3 days, and if the system revalidated the declared LH surge, for example three days later, on the basis of the sustained increase in urinary LH signal and on the rise in P3G, the monitor accuracy will be significantly enhanced. Similarly, if the monitor requested daily urine tests throughout the cycle, the delayed LH surges may have been identified. The daily requirement in testing may even increase the overall compliance since the user may get into a habit of performing the test every day.

This study was designed to assess the pragmatic issue of whether the home-use fertility monitor can reliably replace the existing daily urine and whether it offers increased convenience to women. In a significant number of cycles the monitor indicated high fertility long before the ovulation had occurred and thus, there is really no advantage to providing this advance warning either in a clinical or a research setting. Conversely, as the rise in LH is the most predictable method of determining the days of high fertility (WHO 1980), an error of just around 8% in LH detection can be tolerated. In conclusion, this microelectronic system in detecting the urinary LH peak can be invaluable in contraceptive research because it is easy to use and provides reliable data on compliance. However, further technical adjustments are called for in giving advanced warning of the fertile period (identifying High fertile days).
CHAPTER 6

THE ENDOMETRIAL EFFECTS OF MID-LUTEAL PHASE ADMINISTRATION OF MIFEPRISTONE
6.1 Introduction

Human endometrium is a target organ for the ovarian steroidal hormones oestradiol and progesterone. One of the fundamental roles of progesterone is the differentiation of an oestrogen-primed endometrium (Corner & Allen 1929). The endometrial receptivity that permits successful implantation depends upon timed and regulated synthesis and secretion of a specific set of progesterone-induced proteins in an oestrogen-primed endometrium. If a pregnancy fails to occur, the corpus luteum regresses with a subsequent fall in progesterone levels. There is now compelling evidence that a period of exposure of the oestrogen-primed endometrium to progesterone, followed by a withdrawal of progesterone are the hormonal prerequisites for menstruation (Critchley et al. 2001). The characteristics of the endometrial changes (including the extensive changes observed in the endometrial vasculature) associated with the withdrawal of progesterone and menstrual bleeding, suggest an involvement of vaso-active local mediators. Prostaglandin (PG) activity in the endometrium is modulated by progesterone, and their widely recognised vasoactive properties make PGs to be prime candidates for mediators of progesterone action on the endometrium (Baird et al. 1996).

Prostaglandins (PGs) are synthesised from arachidonic acid (AA) thus, the liberation of AA from precursors (present as membrane bound phospholipids) by the action of phospholipase A_2 (PLA_2) is one of the first and a rate limiting steps in PG synthesis (Bonney et al. 1987 a & b; reviewed in Poyser 1992). AA is then converted to prostanoids including PGE_2, PGF_2a, and PGI_2 by the actions of cyclo-oxygenase (Figure 6.1). In the endometrium, PGs are not stored, but immediately synthesised and released, and metabolised to inactive metabolites by prostaglandin 15 dehydrogenase (PGDH) enzyme (Ensor et al. 1990).

The antiprogesterone compound mifepristone (RU 486; Roussel Uclaf, Paris, France) is a synthetic 19-norsteroid with a specific high affinity for binding to the progesterone receptor. It blocks the biological effects of progesterone at the level of the progesterone
Pathways for AA metabolism in the uterus.

Figure 6.1 Pathway of Arachidonic acid metabolism in the Uterus
Predominating in: ■ endometrium □ myometrium

receptor (Spitz & Bardin 1993). In the mid-luteal phase, mifepristone at a single dose of 50 - 800 mg, induces menstrual bleeding within 72 hours (Schaison et al. 1985; Shoupe et al. 1987). Luteolysis was incomplete in two thirds of the subjects, and they experienced a further episode of vaginal bleeding at the expected time of menses. In the remainder there was complete luteolysis with only one episode of bleeding. Thus the vaginal bleeding observed after mifepristone without a decrease in the circulating progesterone values, appears to be due to a direct effect on the endometrium. Johannisson et al. in 1988 suggested that the menstrual bleeding induced by mifepristone in mid-luteal phase to be a direct endometrial vascular effect. Following treatment with 50 mg of mifepristone in the mid luteal phase (days 20 - 23), they
described a significant reduction in the capillary luminal area and diameter associated with degenerative changes in the endothelial cells, which preceded the menstrual shedding. These changes did not always accompany regressive changes in the adjacent stroma. This effect of mifepristone on the endometrium (at a time when the endometrial progesterone receptor level is relatively low) is poorly understood, and has been hardly investigated.

The endometrial effects of early luteal phase administration of antiprogesterones have been extensively investigated (Swahn et al. 1990; Gemzell-Danielsson et al. 1996; Cameron et al. 1996). In the early luteal phase mifepristone inhibits progesterone induced down-regulation of PR and oestrogen receptors while antagonising the progesterone action on endometrial markers such as prostaglandin dehydrogenase (PGDH), which are known to be progesterone dependent (Casey et al. 1980; Greenland et al. 2000). Moreover, PGDH has been postulated as a useful marker of the “closure” of the implantation window, and the effect of mid-luteal administration on such markers might add to our current understanding of potential contraceptive actions of mifepristone.

Our study investigated the mechanism of mifepristone-induced endometrial shedding & vaginal bleeding in the endometria of 16 healthy women with regular cycles. The endometrial biopsies were performed between 0 (control), and 6 to 48 hours following mid-luteal phase administration of a single dose 200 mg of mifepristone. We examined the expression and the distribution of sex steroid receptors in the endometrium and the expression of PGDH, and cyclo-oxygenase – 2 (COX-2). Alterations in the expression of such progesterone-dependent proteins may widen our understanding of the mechanism by which mifepristone induces endometrial bleeding in the mid-luteal phase.
6.2 Materials & Methods

6.2.1 Subjects
Initially, 20 healthy women with regular cycles (25 - 30 days duration) aged between 26 and 42 years (mean age 34 years) were recruited in to a randomised, single centre study with mifepristone. Women were either using a reliable non-hormonal method of contraception or were abstinent. All women underwent a comprehensive screening procedure before commencing the study. This consisted of a full medical history and routine physical and gynaecological examination, together with measurement of blood pressure, pulse, height and weight. In addition, a venous blood sample was taken for full blood count, serum biochemistry and liver function. These blood tests were repeated at the end of the study. All women kept a menstrual diary card, and recorded all vaginal bleeding experienced during the study period and in the following cycle, and the day in which they identified an LH surge using urinary dipsticks.

We also studied the endometrial samples from four women taking part in a separate study, which evaluated the secretory endometrium. Those women also used the same urinary dipsticks to identify the urinary LH surge and the biopsies were collected 7 or 8 days after the first day of the urinary LH surge. Since these women did not receive any treatment the four biopsies were included in to our control group. Therefore, the total number of biopsies analysed in the control group was seven. Lothian Research Ethics Committee approved the study and, informed written consent was obtained from each woman.

6.2.2 Study Design
The women were monitored over two consecutive cycles; a treatment and follow-up cycle. Each of the 20 women in the study were allocated to the next consecutive study number in the randomisation list before commencing the study. The randomisation list was produced using SPSS such that each study number was randomly assigned to one of five groups in the study. The list was balanced after each block of 5.
Women used LH detection kits (Oviquick, Unipath, Bedford, UK) to detect the Luteinizing Hormone surge in a first sample of urine. 16 women took 200mg of mifepristone on day 8 after the onset of the urinary luteinizing hormone surge (LH+8). An endometrial biopsy was obtained at 6, 24, 36 or 48 hours after taking mifepristone. In order to assess the steroid receptor expression and endometrial parameters of the normal secretory endometrium in the mid-luteal phase, four women were randomly allocated to a control group. They did not take mifepristone, but had an endometrial biopsy 8 days following the identification of the LH surge. On the occasion when the endometrial biopsy was taken, a blood sample was also collected for serum progesterone measurement by radio-immunoassay (See Chapter 7).

Four women were subsequently withdrawn from the study. One due to previously undiagnosed cervical stenosis; in one woman RIA could not confirm the self-detected LH peak; the endometrial samples were inadequate for analysis in two other women. Therefore, we analysed the endometrial samples in 13 women after taking mifepristone in the mid-luteal phase (n = 3, at 6 hours; n = 4, at 24 hours; n = 3, at 36 hours; n = 3, at 48 hours; after mifepristone). Three samples from our original control group, and the above mentioned four additional samples from a separate study made the total number in the control group to be 7.

6.2.3 Detection of the urinary LH peak
The timing of the urinary LH surge were detected by the subjects themselves using a commercially available LH detection kit (Oviquick, Unipath, Bedford, UK), which they used according to the manufacturer’s instructions. Self-detected urinary LH peak was subsequently confirmed by radio-immunoassay (See Chapter 7 - Methods).

6.2.4 Serum progesterone
A blood sample was collected immediately before the endometrial biopsy in all women, stored, and later assayed for progesterone level (methods - Chapter 7). Serum
progesterone measurements were done by using the Coat-A-Cont progesterone procedure (a solid-phase Radio Immuno Assay).

6.2.5 Endometrial biopsies
Endometrial biopsies were obtained using a Pipelle endometrial sampling device (Prodimed, Neuilly-en-Thelle, France) without the need for cervical dilatation or general anaesthetic and were fixed immediately in 4% para-formaldehyde for 24 hours, routinely processed, embedded in paraffin, and sections were cut to 5 μm thickness. All tissue samples were labelled with a code number for anonymity, and except for this number, the mounted sections did not contain any other information.

6.2.5 Immunohistochemistry
Immuno-histochemical staining was performed for immuno-localisation of:

i) Progesterone receptor (PR) with mouse monoclonal anti-human PR antibody (Novocastra Laboratories, Newcastle upon Tyne, UK).

ii) Oestrogen receptor (ER) with mouse monoclonal anti-human ER antibody ER1D5 (DAKO Laboratories, High Wycombe, UK).

iii) Androgen receptor (AR) with monoclonal mouse anti-human AR antibody (F-39, Biogenex antibody, A Merarini Diagnostics, UK).

iv) Prostaglandin dehydrogenase (PGDH) with rabbit polyclonal antibody (Dr H.H.Tai, University of Kentucky, Lexington, USA).

v) Cyclo-oxygenase type 2 (COX-2) with goat polyclonal anti-human COX-2 antibody (Santa Cruz, Biotechnology, UK).

Immunohistochemistry procedures
All protocols were optimised to determine the correct conditions for maximum specific staining, and all sections used as negative controls did not show immunostaining. Each immunostaining procedure was performed in a single run.

(Chapter 7 – Methods).
6.2.6 Scoring and immunohistochemistry analysis

We employed a semi-quantitative subjective scoring system to evaluate the intensity and the localization of immunoreactivity in entire tissue sections. Previously we have reported that the immunostaining patterns in endometrial sections measured by the subjective semi-quantitative scoring, showed an almost perfect correlation (regression coefficient of 0.963) with that measured objectively by computerized image analysis (Wang et al. 1998). Therefore, the less time consuming, semi-quantitative scoring system provides a valid score suitable for graphical presentation.

Two independent observers using light microscopy visually assessed all coded sections. The two separate scores were then compared to obtain a more objective final score for each section. Once the final score had been agreed for all sections in the five-immunostaining runs, the code was broken. Afterwards the final immuno-staining scores were analysed by the respective groups.

The immuno-staining intensity of the steroid receptors (PR, ER, and AR) were scored using a four point scoring scale, where the intensity of staining was assigned as 0 = none, 1 = weak, 2 = distinct, 3 = strong. However, the staining intensity of PGDH and COX-2 showed a narrow range and therefore we adapted a three point scoring scale where the score of zero indicated an absence of immuno-reactivity; 1 = faint immuno-reactivity; 2 = strong immuno-reactivity.

6.2.7 Statistical analysis

Since mifepristone induced bleeding by 36 – 48 hours in all women, a preliminary analysis was performed to determine the appropriate statistical test for analysis of the data. The mean staining intensity scores between 6 hour and 24 hour groups and between 36 hour and 48 hour groups showed no significant difference, hence the 13 samples after treatment with mifepristone were analysed in two groups; group 1 (6 to 24 hours after mifepristone n = 7) and group 2 (36 to 48 hours after mifepristone n = 6). Comparisons
between these two groups, was tested by non-parametric Kruskal-Wallis ANOVA test, and the Dunn's Multiple Comparisons Test since they were discontinuous data sets.

6.3 Results

All women reported vaginal bleeding commencing 36 - 48 hours after taking mifepristone. Four women in group 2 (one woman after 36 hours, and three women after 48 hours) had already started to bleed at the time the endometrial biopsy was taken, the others started bleeding after the biopsy. The bleeding lasted for 12 - 72 hours, and all but three women reported a second bleed at the time of the expected menses. In these three women, a second episode of bleeding occurred approximately four weeks later.

6.3.1 Serum Progesterone

Treatment with mifepristone had significantly reduced serum progesterone levels in all women when compared with the controls (13.2 nmol/L vs. 34.8 nmol/L, p = 0.001), but the levels between 6-24 h group and 36-48 h group were not statistically significant (p = 0.32).

6.3.2 PGDH immunoreactivity

Intense PGDH immunoreactivity was observed in the cytoplasm of predominantly glandular epithelium (with a lesser degree of staining in the stromal cells) in all mid-luteal phase control sections. The abundance of PGDH positive immunostaining clearly declined to be virtually absent by 36 - 48 hours in both glands and in stroma. The difference in PGDH staining scores between the control group and 36 - 48 h group were significant (p = <0.05) (Figures 6.2 and 6.3).
**Endometrial effects of mid-luteal mifepristone**

**Figure 6.2 PGDH immuno-staining**

a. Negative control = Mid-luteal endometrium.

b. PGDH immuno-staining in midluteal phase endometrium, demonstrating positive immuno-reactivity in the glands and stroma.

c. Endometrium biopsied 6-24h after mifepristone, showing mild decrease in immunostaining in both compartments.

d. Note the clear decrease in immuno-staining in both glandular and stromal compartments of the endometrium biopsied 48h after taking mifepristone on LH+8.

*Scale bar, 50 µm.*
Figure 6.3

Intensity of staining for PGDH (a) and COX-2 (b) in endometrial glands (■) and stroma (□) in the mid-luteal phase of the cycle before (control) and at times after administration of 200mg mifepristone.

Bar. Median and range
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Figure 6.4  **COX-2 Immunostaining**

a. Negative control = mid-luteal endometrium.

b. COX-2 immuno-staining in an endometrium collected in the mid-luteal phase illustrating negligible immuno-staining in both glands & stroma.

c. No obvious change in the endometria for COX-2 immuno-reactivity biopsied 6-24h following mifepristone.

d. Endometrium biopsied 36-48h after mifepristone, on LH+8 showing a marked increase in immuno-staining in the glandular cytoplasm.

*Scale bar, 50 μm.*
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Figure 6.5  PR immuno-staining

a. Mid-luteal endometrium, negative control.

b. A control biopsy displaying faintly positive PR immuno-reactivity confined to the nuclei of both glandular epithelial and stromal endometrial cells in the mid-luteal phase (LH+8) endometrium.

c. The weak glandular PR staining in an endometrial sample biopsied 6-24h after mifepristone.

d. Glandular PR immuno-staining appears to decline further by 36 - 48 hour after mifepristone. Note the persistence of the weak stromal staining across the groups.

Scale bar, 50 μm.
**Endometrial effects of mid-luteal mifepristone**

- **a. Negative control**
- **b. Control (0 h) sample at LH+8**
- **c. 6–24 h after mifepristone**
- **d. 36-48h after mifepristone**

**Figure 6.6  ER immuno-staining**

a. Negative control = mid-luteal endometrium.

b. Mid-luteal phase control sample, showing weak ER immunostaining in the stroma as well as in the glands.

c. & d. Endometria biopsied 6-24h and 36 – 48h after mifepristone, respectively, showing no obvious change in the staining.

*Scale bar, 50 μm.*
**Endometrial effects of mid-luteal mifepristone**

Figure 6.7  *Androgen receptor immuno-staining*

a. Negative control = Mid-luteal endometrium.

b. Control endometrium biopsied on LH+8, showing the distinct AR immunostaining localized predominantly in the stroma with faint or absent glandular epithelial staining.

c. & d. Endometria biopsied after mifepristone showing no apparent change in the AR immuno-staining.

*Scale bar, 50 μm.*
6.3.3 COX-2 immunoreactivity
In all samples (treated / untreated), COX-2 staining was localised predominantly in the endometrial glands with no (or barely detectable) staining in the stroma (Figure 6.4). Untreated endometria in the mid-luteal phase showed minimal staining for COX-2 in the glandular cellular compartments. After mifepristone, a significant increase in immunoreactivity was apparent at 36 - 48 hours (Figure 6.3) \((p = 0.0018)\).

6.3.4 Steroid receptor immunostaining.
Control biopsies displayed faintly positive PR immuno-reactivity confined to the nuclei of both glandular epithelial and stromal endometrial cells. The weak glandular PR staining appears to decline further by 36 - 48 hour after mifepristone. Stromal staining however remains weak across the groups (Figure 6.5).

In all mid-luteal phase control samples, there was weak ER immunostaining in the stroma as well as in the glands. There was no obvious change in the staining after mifepristone (Figure 6.6).

The distinct AR immunostaining was localized predominantly in the stroma in the control samples with faint or absent glandular epithelial staining, and there was no apparent change observed in the AR immunostaining after the treatment with mifepristone (Figure 6.7).

6.4 Discussion
Endometrial shedding and vaginal bleeding is observed after withdrawal of progesterone (e.g. luteal regression) from an oestrogen primed endometrium that is subsequently exposed to progesterone. Similar bleeding is also seen after the pharmacological withdrawal of progesterone via administering the antiprogestrone, mifepristone in the luteal phase of the cycle. While the endometrial morphology exhibits a marked sensitivity to mifepristone, with 0.5 mg daily dose being the threshold dose for delay in
endometrial maturation (Croxatto et al. 1993; Cameron et al. 1996; Spitz et al. 1996; Gemzell-Danielsson et al. 1997), in general, higher doses (in excess of 10 mg) are required to produce endometrial shedding and menstrual bleeding (Green et al. 1992; Schaison et al. 1985; Shoupe et al. 1987).

All women in our study reported vaginal bleeding commencing at 24 to 48 hours after taking 200 mg of mifepristone in the mid-luteal phase. The mifepristone induced bleeding in the mid-luteal phase, was not dependent on a parallel decline in the circulating progesterone levels, but was due to direct antagonism of the action of progesterone on the endometrium (Swahn et al. 1988). The endometrial shedding at the onset of normal menstruation is due to a fall in progesterone levels that follow luteal regression. In 13 out of the 16 subjects, this was followed by a second bleed of normal character at the expected time of the next menses. Although mifepristone significantly depressed the serum progesterone value in all women, the occurrence of a second bleed in the majority suggest only a partial luteolysis and a direct effect of mifepristone on the endometrium.

The current understanding advocates a central role for PGs as a trigger mechanism for menstruation (Figure 6.8, Baird et al. 1996; Critchley et al. 2001; Milne et al. 2001). The concentration of PG in any tissue is related to its rate of synthesis and metabolism, and the variation in the endometrial release of PGs at different stages of the menstrual cycle suggests an ovarian hormonal influence. The two key-enzymes that control the endometrial PG synthesis (PLA₂ and COX) appear to be under the influence of progesterone. COX enzyme exists as two isoforms produced by two different genes (COX-1 and COX-2). COX-I is constitutively expressed while COX-2 expression is modulated by a variety of stimuli and may be inhibited by progesterone in the endometrium (Jones et al. 1997). PLA₂ also appears to be present in the endometrium in two different iso-forms; the calcium dependent and inducible PLA₂(i) which is localised in the endometrial glands, and the calcium independent PLA₂(ii) that is predominantly
confined to the stroma (Bonney et al. 1987). Conversely, prostaglandin dehydrogenase (PGDH) metabolises PGs to inactive metabolites and this enzyme is induced by progesterone (Casey et al. 1980 & 1993; Kelly et al. 1986). The increase in PGDH activity in a secretory endometrium that occurs in response to the rising levels of progesterone in the luteal phase (Casey & MacDonald 1980) can be prevented by administration of antigestagens shortly after ovulation (Cameron et al. 1997). Thus progesterone is responsible for stimulating PGDH and suppressing COX-2.

**Figure 6.8**  *Vascular origins of endometrial shedding & vaginal bleeding*

The decline in progesterone concentrations (luteal regression) or inhibition of its action (mifepristone) exerts a primary effects on the peri-vascular region surrounding blood vessels (bv). Adapted from; Baird et al. (2000) with permission.

In our study, treatment with mifepristone in the mid-luteal phase resulted in a decreased PGDH and an increased COX-2 expression in the endometrial glands, which was apparent at 36 hours following mifepristone. This effect will be synergistic in increasing
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Endometrial PGs (due to a synchronized suppression of metabolism, with an augmentation in the synthesis), and will lead to increased uterine activity and menstrual bleeding. In addition, progesterone withdrawal mediates the degradation of the endometrial extra-cellular matrix by inducing matrix metalloproteinases (MMP) (Lockwood et al. 1998), and other cytokines (Critchley et al. 2001 & references therein).

Attempts have been made to demonstrate the effects of progesterone on the endometrial PGs activity both in-vitro as well as in-vivo studies. Progesterone appears to enhance the PG biosynthetic capacity of the secretory endometrium. This is demonstrated by the in-vitro studies employing cell culture techniques and by maintaining endometrial explants in culture (Smith et al. 1984; Kelly et al. 1984; Abel & Baird 1980). Conversely, progesterone has been shown to suppress the release of PGs from the endometrium (Abel & Baird 1980; Kelly & Smith 1987). Studies in-vitro have reported a reduction of both the oestradiol-stimulated and the basal PG production by progesterone (Abel & Baird 1980; Kelly & Smith 1987). Furthermore, during pregnancy, when progesterone levels are high, basal endometrial PG production is also reduced (Maathuis et al. 1978). It had been suggested that this effect might involve the inhibitory effect of progesterone on the PLA2 activity (Bonney et al.1987).

The withdrawal of progesterone from an endometrium that has been primed with progesterone and oestradiol results in an increase COX-2 expression, while continuing exposure to progesterone is associated with low levels of COX-2 expression (Critchley et al. 1999). Evidence for increased PG activity by antagonising progesterone also comes from in-vitro data, which showed a dose-dependent induction of PGF2α release from endometrial stromal cells (Kelly et al. 1986) and also from the in vivo observation of increased uterine contractility after mifepristone possibly due to increase PGs (Norman et al. 1991; Gemzell-Danielsson et al. 1994). This is further supported by the inhibition of glandular PGDH expression seen after administration of antiprogestrones in the early luteal phase and during the early pregnancy (Cheng et al. 1993; Cameron et al. 1996).
A decrease in the uterine PGF\textsubscript{2α} release (Gemzell-Danielsson et al. 1994), and in the luminal expression of COX-2 had been reported following mifepristone in the early-luteal phase (Marions et al. 1999). However, in the early-luteal phase progesterone values are relatively low, and as a consequence the PR and ER expression is maximal, while the converse is true for the mid-luteal phase of the cycle. Therefore, in the early luteal phase mifepristone may prevent the effects that are to be exerted by progesterone, where as in the mid-luteal phase it may antagonize the actions of progesterone, which at that time appears to be the suppression of PGs release.

Our results are consistent with previous reports (Cameron et al. 1996; Jones et al. 1997) that demonstrated localisation of PGDH & COX-2 in the glandular epithelium and, also supports the in-vitro evidence that glands to be the main site for PG synthesis (Lumsden et al. 1984; Smith et al. 1988).

In our study, there was weak staining for PR in the nuclei of stromal cells in the control samples with minimal ER staining. AR staining was confined to stroma. There was no change in intensity or distribution of staining for steroid receptors after mifepristone (Figures 6.5, 6.6 & 6.7).

Previous studies have reported an increased expression of PR & ER in both glandular and stromal compartments (increase more pronounced in the glands) in the mid-luteal endometrium after early luteal phase administration of antiprogestergones (Berthois et al. 1991; Maentausta et al. 1993; Cameron et al. 1996). In all these studies the endometrium was sampled at least 48 hours or more later than mifepristone. We did not observe a significant change in the endometrial immunoreactivity for all three steroid receptors (ER, PR, and AR) after mifepristone. PR level in the mid-luteal endometrium is minimal compared to the early luteal phase. This may be a reason why we did not see the same level of progesterone antagonism in steroid receptor expression as seen after the early luteal phase administration. Furthermore, the studies mentioned above described effects
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seen more than 48 hours after the administration of mifepristone hence; their results may not be comparable to our observations. There are further suggestions that the predominant PR subtype in endometrial glands to be different in early and mid luteal phase, and the functional diversity of these receptor subtypes may explain the different results observed (Mote et al. 1999).

Although others have reported barely detectable AR immunoreactivity in the glandular epithelium (Slayden et al. 2001), untreated normal mid-luteal endometria in our study exhibit weak glandular and surface epithelial staining. Immediately before menstruation in the late secretory phase, a decrease in stromal AR immuno-staining had been reported. In contrast, an up-regulation was observed in glands as well as in stroma, following the early luteal phase administration of mifepristone (Slayden et al. 2001). Once more, here the biopsy was taken more than 48 hours after administering mifepristone.

The distinct endometrial effects seen after the mid-luteal administration of mifepristone add to our understanding of the mechanism of menstruation. There is overwhelming evidence that PGs are involved in the process of normal menstruation (Baird 1996 and references therein). Our results show a down-regulation of PGDH expression and a simultaneous up-regulation of COX-2 expression after administering mifepristone in the mid-luteal phase. Therefore, we conclude that mifepristone induces endometrial-bleeding in the mid-luteal phase by a mechanism involving both PGDH and COX-2 to increase local PG levels in the endometrium.
CHAPTER 7

METHODS
7.1 **Hormone assays**

7.1.1 **Serum progesterone assay**

Venous blood was collected, centrifuged to separate serum, and stored serum was later assayed for progesterone using Coat-A-Cont progesterone procedure (solid-phase radioimmunoassay; Diagnostic Products (UK) Ltd., Glyn Rhomwy, Llanbersi, Caernarfon, Gwynedd, North Wales, UK).

7.1.2 **Urinary hormone assays**

Urine samples were frozen and later assayed in batches (with all samples from one subject assayed in a single batch) for measurement of urinary LH, E3G and P3G.

Quantitative assessment of urinary LH was performed using a LH MAI Aclone kit (BIOSTAT-DIAGNOSTICS, Stockport, Cheshire, UK). This method incorporates two high affinity monoclonal antibodies into an immuno-radiometric assay system and offers a working range of 1.5 – 200 mIU/ml. Urinary P3G was measured using a direct enzyme immunoassay (sensitive working range 0.16 ug/L – 20 ug/L), while direct immunoassay was used to measure E3G levels (working range 8.36 nmol/L - 2140 nmol/L). Intra-assay coefficients of variation were 6% for E3G, 10% for P3G, and 3% for LH (Yong et al. 1992). Geometric means of daily replicates were divided by the respective daily creatinine concentration to correct for variations in the dilution of the urine specimen.

7.2 **Statistical Analysis**

Both pragmatic and explanatory studies were undertaken as a part of this thesis. For example, studies that evaluated the mechanism of action of LNG and vaginal bleeding (mifepristone induced) belong to the explanatory category while the feasibility of administration of mifepristone, as a once-a-month pill was a pragmatic trial. During the pragmatic trials we tried to mimic real life yet close monitoring and frequent laboratory tests were required during the explanatory studies. The following statistical tests were used for statistical analysis of the results.
7.2.1 **t-test**
Employing the t-test assessed a statistically significant difference between two normally distributed sample means (example, Chapter 2).

7.2.2 **Confidence intervals**
A significance test and a p-value do not explain the precision of the estimated treatment effect. Quoting a confidence interval, or the range of possible differences with which the data were consistent, allowed an assessment of both the statistical and clinical significance (example, Chapter 4).

7.2.3 **Kruskal-Wallis ANOVA test**
When the normal distribution with uniform variance cannot be assumed in a data set, Kruskal Wallis ANOVA test was used to analyse the variances by ranks (example, Chapter 6).

7.2.4 **Correlation**
In chapter 4, statistical testing on correlation was done on data aggregated to patient level. Thus, for example, correlating each patient’s age with the percentage compliance over all her cycles tested the association between age and compliance. Since both of these variables were quantitative and not very skewed we felt Pearson’s correlation was appropriate. Similarly, for binary factors such as whether or not a patient had had previous children, the percent compliance was compared between these two patient groups using a two-sample t-test, which is in fact identical to the Pearson correlation. Clearly chi-squared or Fisher tests are not applicable in this type of analysis where a continuous outcome is being used.

7.2.5 **The Dunn’s multiple comparisons test**
This multiple comparison procedures provided a way to compare and identify the differences in the means of discontinuous data sets with dissimilar numbers in each group (example, Chapter 4).
7.2.6 Mann-Whitney U test
Apparently skewed data were analysed with a non-parametric Mann-Whitney U test (for example, in Chapter 2 the comparison of the difference between urinary E3G levels on the day of LNG treatment in cycles that had delayed LH peak and the 8 cycles that did not).

7.3 Endometrial collection and processing
7.3.1 Endometrial sampling
Both anterior and posterior uterine endometrium was sampled using a Pipelle endometrial sampling device (Prodimed, Neuilly-en-Thelle, France). This disposable, plastic device is 3 mm in diameter and can be easily introduced through the cervical canal into the uterine cavity with minimal discomfort to the un-anaesthetised woman. Once the device has reached the uterine cavity, suction can be created within the device cavity allows the aspiration of a small endometrial sample.

7.3.2 Tissue fixation, processing and microtomy
Tissue samples were cut into small pieces (to aid faster fixation), fixed immediately in 4% paraformaldehyde, for 24 hours at 4°C. This fixation preserves the intracytoplasmic protein structure, by forming cross-linking bridges. The tissue biopsy was then stored in 70% ethanol before routinely wax embedding in a Leica TP 1050 (Leica UK Ltd, Milton Keynes, UK). Once the fixation was complete, the tissue was dehydrated (via passage through a ascending grades of alcohol) and was embedded in paraffin wax. 5 μm thick sections were cut from the paraffin blocks using a microtome (HM 325, Shandon, Cheshire, UK), and the sections were dried on glass slides (Superfrost plus, BDH, UK) at 37°C overnight.

7.4 Immunohistochemistry
An avidin Biotin Peroxidase Complex Method (ABC) of antigen detection was utilised for the immuno-staining protocols. This method utilises the high affinity of avidin for biotin. After the addition of an antigen specific primary antibody, a biotinylated secondary antibody (where biotin has been covalently linked to the antibody) was used to link the primary antibody to a subsequently applied complex
of avidin, biotin, and the enzyme horseradish peroxidase (ABC complex). Since avidin has a high affinity and 4 binding sites for biotin, unoccupied sites on the avidin of the ABC complex bind to the biotin of the biotinylated secondary antibody. The reactions were visualised by the addition of the peroxidase substrate, hydrogen peroxide (H₂O₂) and 3, 3' Diaminobenzidine Tetrahydrochloride (DAB). DAB acts as a chromogen and is oxidised during the enzyme-substrate reaction to produce an insoluble dark brown end product.

7.4.1 PR immunostaining protocol

Tissue sections were dewaxed in Histoclear (National Diagnostics, Yorkshire, UK), and rehydrated in descending grades of ethanol in water. Then they were washed in distilled water, and 0.01M phosphate buffered saline (PBS tablets, Sigma, Dorset, UK, pH 7.4 - 7.6) for 10 minutes. Tissue sections were microwaved at full power in 0.01M Sodium Citrate buffer (pH 6.0) for 10 minutes and then allowed to stand in the microwave oven for further 20 minutes, as an antigen retrieval measure. In order to block non-specific endogenous peroxidase activity, following another wash with PBS, the sections were incubated in 3% hydrogen peroxide in distilled water for 10 minutes at room temperature (RT). After a further wash in PBS, a non-immune block was performed, by incubating the tissue sections in normal horse serum (Vector Laboratories, Peterborough, UK) for 20 minutes at RT. The sections were then incubated with a mouse monoclonal anti-human PR antibody at a dilution of 1:40 (0.88 µg/ml protein; Novocastra Laboratories, Newcastle upon Tyne, UK). Mouse IgG at a matching immunoglobulin concentration as the primary antibody (1:6000 dilution) was substituted for the anti-human PR antibody on negative control sections and from this step forward negative control sections were washed separately. Late-proliferative endometrium was included as a positive control tissue, and it is known to express high levels of PR. Following the primary antibody binding, sections were washed in Phosphate Buffered Saline with Tween (PBST). Thereafter the tissue sections were incubated with biotinylated horse anti-mouse IgG (Vectastain, Vector laboratories, Peterborough, UK) at RT for 30 minutes. After a further 10 minutes of washing in PBST, sections were incubated at RT for 30 minutes with a complex of avidin and biotin coupled to a horseradish peroxidase enzyme (Vectastain Elite,
Vector Laboratories, Peterborough, UK). Sections were washed again before the antigen bound ABC-HRP enzyme was identified by applying 3,3'-diaminobenzidin in hydrogen peroxide (DAKO liquid DAB substrate, DAKO Laboratories, High Wycombe, UK). Sections were then washed in water before counterstaining with the non-specific nuclear stain, Harris' Haematoxylin (Pioneer Research Chemicals Ltd, Essex, UK). Finally, sections were washed in scott's tap water, dehydrated by passage through graduated ethanol of increasing concentration, cleared by immersing in Xylene for at least 30 minutes and were mounted in Pertex (Cellpath, Hemel Hampstead, UK).

Similar protocols were employed for oestrogen receptor (ER), androgen receptor (AR), Prostaglandin dehydrogenase (PGDH), and Cyclooxygenase-2 (COX-2). Specific features for each protocol were as follows.

7.3.3 ER immunostaining protocol
Primary monoclonal anti-human ER antibody ER1D5 (0.58 µg/ml protein; DAKO Corp. Laboratories, High Wycombe, UK) was used at dilution of 1:400. Non-immune mouse IgG at the equivalent concentration (1:2400) was used in place of the primary antibody in the negative controls. Sections were incubated with the secondary antibody and ABC complex (Vectastain Elite, Vector Laboratories, Peterborough, UK) for 1 hour. Mid proliferative endometrial sections were included as positive controls.

7.3.4 AR immunostaining protocol
Sections were subjected to a different antigen retrieval step, in which they were pressure cooked (Tefal, Nottingham, UK) in 0.01M Sodium Citrate buffer (pH 6.0) for 5 minutes and then were allowed to rest in buffer for a further 20 minutes. After the wash that followed the endogenous peroxidase block, slides were incubated with Avidin for 15 minutes followed by Biotin for 15 minutes (Avidin-Biotin blocking kit, Vector Laboratories, Peterborough, UK) with a PBS wash in between, to block endogenous Biotin activity. Slides were then incubated overnight at 4°C with monoclonal mouse anti-human AR antibody (F 39, BioGenex Laboratories, Inc.)
antibody, A Merarini Diagnostics, Berkshire, UK) at a 1:480 dilution in PBS/BSA/Gelatin gel (see recipes, table 7.2). Sections were incubated with the secondary antibody and ABC complex for 1 hour. Mouse IgG at a 1:600 dilution was used for negative control sections, and mid proliferative endometrium was included as a positive control.

7.3.5 PGDH immunostaining protocol
A polyclonal rabbit anti-human PGDH antibody (Dr H. H. Tai, University of Kentuckey, Lexington, USA) was used to identify PGDH immunoreactivity with an avidin-biotin peroxidase detection system. An antigen retrieval step was not required to expose this cytoplasmic epitope. Slides were exposed to a non-immune block by incubating in 20% normal goat serum (SAPU, BSA grade) for 20 minutes. Sections were then incubated with the primary antibody at a 1:3000 dilution overnight at 4\(^0\) C, and rabbit IgG at 1:6000 dilution, substituted the primary antibody for negative controls. Biotinylated goat anti-rabbit was applied for 30 minutes at RT followed by the ABC-complex (Vectastain, Elite ABC Kit, Vector Laboratories, Peterborough, UK) for 60 minutes at RT. Late 3\(^{rd}\) trimester fetal membranes was included as a positive control.

7.3.6 COX-2 immunostaining protocol
Detection of COX-2 immunoreactivity employed a polyclonal goat anti-human COX-2 antibody (0.3 \(\mu\)g/ml protein; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) with the avidin-biotin peroxidase detection system. Endogenous peroxidase activity was quenched with 3% \(\text{H}_2\text{O}_2\) in methanol for 30 minutes at RT. An antigen retrieval step was conducted when the sections were pressure cooked in 0.01 M Sodium Citrate buffer (pH 6.0) for 2 minutes and then allowed to rest in the pressure cooker (Tefal, Nottingham, UK) for a further 20 minutes. Incubating in avidin and biotin blocked endogenous biotin, as described for the AR immunostaining protocol. A non-immune block was conducted by incubating the sections in 20% normal rabbit serum (Diagnostics Scotland, UK) for 20 minutes, followed by a 1 hour incubation of the sections with the primary antibody at a 1:600 dilution at 37\(^0\) C. Goat IgG at a 1:3000 dilution (matching IgG concentration as primary antibody) was substituted.
for negative control slides. Sections were then incubated in a 1:500 dilution of biotinylated horse anti-goat antibody (Vector Laboratories, Peterborough, UK) for 30 minutes at RT followed by the ABC-complex (DAKO Laboratories, High Wycombe, UK) for 20 minutes at RT. Menstrual endometrium was included as a positive control tissue.

7.4.6 Scoring and immunohistochemistry analysis

We employed a semi-quantitative subjective scoring system to evaluate the intensity and the localization of immunoreactivity in entire tissue sections. Previously we have reported that the immunostaining patterns in endometrial sections measured by the subjective semi-quantitative scoring, showed an almost perfect correlation (regression coefficient of 0.963) with that measured objectively by computerized image analysis (Wang et al. 1998). Therefore, the less time consuming, semi-quantitative scoring system provides a valid score suitable for graphical presentation.

Two independent observers using light microscopy visually assessed all coded sections. The two separate scores were then compared to obtain a more objective final score for each section. Once the final score had been agreed for all sections in the five-immunostaining runs, the code was broken, then the final immuno-staining scores were analysed.

The immuno-staining intensity of the steroid receptors (PR, ER, and AR) and COX-2 were scored using a four point scoring scale, where the intensity of staining was assigned as 0 = none, 1 = weak, 2 = distinct, 3 = strong. However, the staining intensity of PGDH showed a narrow range and therefore we adapted a three point scoring scale where the score of zero indicated an absence of immunoreactivity; 1 = faint immunoreactivity; 2 = strong immunoreactivity.
### Table 7.1 Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Normal Serum</th>
<th>Primary antibody</th>
<th>Dilution of Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>Horse</td>
<td>Mouse monoclonal anti-human PR antibody (Novocastra Laboratories, Newcastle upon Tyne, UK)</td>
<td>1:40</td>
<td>Biotinylated horse anti-mouse</td>
</tr>
<tr>
<td>ER</td>
<td>Horse</td>
<td>Mouse monoclonal anti-human ER antibody ER1D5 (DAKO Laboratories, High Wycombe, UK)</td>
<td>1:400</td>
<td>Biotinylated horse anti-mouse</td>
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<tr>
<td>AR</td>
<td>Horse</td>
<td>Monoclonal mouse anti-human AR antibody (F-39, BioGenex antibody, A Merarini Diagnostics, UK)</td>
<td>1:480</td>
<td>Biotinylated horse anti-mouse</td>
</tr>
<tr>
<td>PGDH</td>
<td>Goat</td>
<td>Rabbit polyclonal antibody (Dr H.H.Tai, University of Kentuckey, Lexington, USA)</td>
<td>1:3000</td>
<td>Biotinylated goat anti-rabbit</td>
</tr>
<tr>
<td>COX-2</td>
<td>Rabbit</td>
<td>Goat polyclonal anti-human COX-2 antibody (Santa Cruz, Biotechnology, UK)</td>
<td>1:600</td>
<td>Biotinylated horse anti-goat</td>
</tr>
<tr>
<td>Recipe</td>
<td>PBS tablets (Sigma, UK)</td>
<td>Distilled water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-------------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) pH 7.4 - 7.6</td>
<td>5 tablets</td>
<td>1 litre</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffered Saline with Tween (PBST) pH 7.4 - 7.6</td>
<td>PBS as above</td>
<td>1 litre</td>
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<tr>
<td></td>
<td>Sodium chloride</td>
<td>8 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween 20</td>
<td>100 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS/BSA/gel</td>
<td>Sodium dihydrogen orthophosphate 1-hydrate 0.345 g, Sodium chloride 0.405 g, Gelatin 0.05 g, Bovine serum albumin (Fraction V) 0.5 g, 2 g Potassium hydrogen carbonate, and distilled water 50 ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scotts tap water</td>
<td>Magnesium sulphate 7-hydrate 20 g, Water 1 litre</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 8

CONCLUSIONS
The main focus of the studies undertaken as a part of this thesis was to evaluate means to advance contraceptive research methodology, while broadening our current understanding of the anti-fertility potentials of two progesterone receptor modulators: antiprogesterone compound mifepristone, and the synthetic progestin LNG.

When LNG was administered twice at a dose of 0.75 mg to 12 healthy female volunteers in the fertile period (immediately before ovulation) of the menstrual cycle, it aborted the LH peak, delayed ovulation and lengthened the cycle in four women (25%). One woman did not ovulate at all despite having an LH surge 2 days after taking LNG. Seven women had apparently normal ovulatory cycles after taking LNG. It is apparent that different women respond differently to the administration of LNG. In all twelve volunteers the luteal phase was significantly shortened following treatment with LNG as compared with the placebo cycles (mean length 11.5 days (SD +/-1.8) vs. 12.9 days (SD +/-2.5) p = 0.005). The eight women in whom pre-ovulatory LNG did not affect the cycle length, showed a significant (p = 0.01) decrease in total LH in the treatment cycles (18.66 U/mol creatinine (SD +/-8.9)) as compared with the placebo cycles (27.08 U/mol creatinine (SD +/-13.57)). We suggest that LNG taken immediately before ovulation acts as an EC by delaying or preventing ovulation. It may also have an effect on luteal function. Other plausible actions of LNG including retardation of the endometrium, interfering with sperm motility and altering cervical mucus may be important, and need to be explored further.

The limiting factor in the once-a-month approach to administering anti-progesterone as a contraceptive pill is the accurate detection of the LH peak. Clearly, the failure to detect the LH peak accurately has a big impact on the overall effectiveness of the method. The home-use fertility monitor provided us with an opportunity to overcome these problems. In our own control group, if sexual intercourse took place on a fertile day the probability of a pregnancy was 0.25 - 0.32. Thirty two women in the treatment group contributed to a total of 178 cycles and there were two pregnancies (probability of pregnancy 0.01). Imperfect use of the system accounted for failure to identify an LH surge in 11.8% cycles while 7.9% were due to monitor
method failure. During the course of the study we developed an algorithm for the administration of mifepristone if an LH surge was not identified. In nineteen exposure cycles (out of 28 cycles in which an LH surge was not identified) mifepristone was administered using this algorithm and there were no pregnancies. Given that in real life the available methods detecting LH surge are not 100% accurate, such an algorithm will be essential to time the administration of mifepristone when an LH surge was missed. Treatment with mifepristone in the early luteal phase did not disrupt the cycle length but women reported slight vaginal bleeding in 15% of the cycles. Also in our study, mis-timed administration of mifepristone led to predictable effects. Therefore, the combination of a home-use fertility monitor with once a month administration of mifepristone (especially if mifepristone is administered at the early luteal phase) is an acceptable contraceptive option with minimal side effects. Unfortunately, it is difficult to envisage how an easier way of defining the correct timing, which required less compliance, could be devised.

Poor compliance can make the results of a study invalid, yet is commonly overlooked in contraceptive research. We were able to compare the incidence of non-adherence detected by a home use fertility monitor with the traditional self-reported incidence of compliance in a group of women who took part in a trial assessing the efficacy of a novel method of contraception. Women failed to perform 24.6% of the tests in the 162 cycles analysed. In 42% of the cycles they missed tests during a critical period, which could have increased their risk of an unwanted pregnancy. During this critical period, women missed fewer tests and admitted to missing even less. Poor compliance was associated with younger women, those who discontinued the study before completion, and cycles where women were not at risk of pregnancy. The use of microelectronic monitoring systems may improve our understanding of the extent of the problem of patient non-compliance, providing objective information that no other monitoring technique can produce. This understanding provides the opportunity to make the optimum use of potentially effective treatments while validating research evidence.
Conclusions

A reliable and simple means to predict the potentially fertile period can assist in contraceptive research in monitoring frequently changing hormone levels, and in identifying the fertile period. In a significant number of cycles the home use fertility monitor indicated high fertility long before the ovulation had occurred and thus, there is really no advantage to providing this advance warning either in a clinical or a research setting. Conversely, as the rise in LH is the most predictable method of determining the days of high fertility, an error of just around 8% in LH detection can be tolerated. Women missing about 19% of the urine tests requested by the monitor is obviously significant, and the monitor offered us an important methodological advance in providing reliable data on the incidence of non-compliance with urine testing, and exactly when a test was missed. Therefore, this microelectronic system in detecting the urinary LH peak can be invaluable in contraceptive research because it is easy to use and provides reliable data on compliance.

The distinct endometrial effects seen after the mid-luteal administration of mifepristone may add to our understanding of the mechanism of menstruation. There is overwhelming evidence that PGs are involved in the process of normal menstruation. All women in our study reported vaginal bleeding commencing at 24 to 48 hours after taking 200 mg of mifepristone in the mid-luteal phase. In 13 out of the 16 subjects, this was followed by a second bleed at the expected time of the next menses. Although mifepristone significantly depressed the serum progesterone value in all women, the occurrence of a second bleed in the majority (81.25%) suggested only partial luteolysis and a direct effect of mifepristone on the endometrium. Our results show a down-regulation of PGDH expression and a simultaneous up-regulation of COX-2 after administering mifepristone. Therefore, we conclude that mifepristone induces endometrial-bleeding in the mid-luteal phase by a mechanism involving both PGDH and COX-2 to increase local PG levels in the endometrium. However, these preliminary data require to be confirmed by a larger study in the future.

In summary, the studies carried out as part of this thesis have demonstrated the importance of understanding the basic mechanisms of progesterone receptor
modulators in various aspects of contraceptive research. They also confirmed the contraceptive potential of mifepristone as a once-a-month pill, and demonstrated that levonorgestrel acts as an EC mainly through directly or indirectly inhibiting ovulation. Moreover, these studies have established the importance of patient non-compliance while recommending a potential methodological modification to detect the incidence of non-compliance in contraceptive research. Finally, the study that evaluated mifepristone induced bleeding, broadened our current understanding of the mechanism of endometrial bleeding by illustrating the synergistic changes on PG synthetic and catabolic enzymes that may produce an increase in the local endometrial concentration of PGs.


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Appendix

Published Papers

The following papers have been published based on the text of this thesis;

Original Articles.


The effects of peri-ovulatory administration of levonorgestrel on the menstrual cycle

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Abstract

Levonorgestrel (LNG) 0.75 mg administered 12 h apart within 72 h of unprotected coitus, is an established method of emergency contraception (EC). The mechanism of action of LNG used in this manner is unknown. We administered LNG 0.75 mg twice immediately before ovulation, to test the hypothesis that LNG acts as an emergency contraceptive by abolishing the pre-ovulatory luteinizing hormone (LH) surge and thereby delaying ovulation. Twelve women took LNG on or before the day of the first significant rise in urinary LH in 12 cycles. In four women, the LH peak and the onset of next menses were significantly delayed (delay of 16.8 days (SD ± 8.7) from the day of mean LH peak in placebo cycles). One woman did not ovulate at all, despite a normal LH peak and cycle length. In the remaining eight women, LNG did not affect ovulation or the cycle length, but the length of the luteal phase and the total luteal phase LH concentrations were significantly reduced. We suggest that LNG acts as an emergency contraceptive by other mechanisms as well as delaying the LH surge and interfering with ovulation. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Emergency contraception; Levonorgestrel; Mechanism of action

1. Introduction

Levonorgestrel (LNG) 0.75 mg administered twice with the two doses 12 h apart has been shown to be an effective method of emergency contraception (EC) when used within 72 h of unprotected intercourse [1,2]. Although the regimen is now licensed in the UK, USA, and throughout much of Europe and is widely regarded as the emergency contraceptive method of choice [3], the mechanism of action remains unknown. The mechanism of action of the Yuzpe regimen of emergency contraception (ethinyl estradiol 100 mcg and 0.5 mg LNG, two doses 12 h apart [4–6], is also incompletely understood but there is good evidence that it delays or inhibits ovulation in at least some cycles [5]. In the WHO study, the efficacy of both LNG and the Yuzpe regimen decreased with time after intercourse [7] and both regimens had a similar effect on the timing of the subsequent menses, suggesting that the mechanism of action of the two regimens may be similar. It has also been shown that large amounts of synthetic progestogens abolish the mid-cycle luteinizing hormone (LH) surge leading to anovulation and delaying the onset of the subsequent menses [8–11].

To test the hypothesis that it acts as a post-coital agent by abolishing the pre-ovulatory LH surge and by delaying ovulation, we administered LNG 0.75 mg twice to 12 healthy female volunteers in the fertile period (immediately before ovulation) of the menstrual cycle and investigated the effects on the timing of ovulation and of the next menses, bleeding patterns; ovarian activity, and LH concentrations.

2. Materials and methods

This was a prospective, randomized, double-blind, cross-over study undertaken in one center. Twelve healthy women (mean age 33.3; range 26–41 years) with regular cycles (mean 27.6 days; range 25–30 days) and mean BMI 25.7 (range 20–34) were recruited. They were all using a reliable non-hormonal method of contraception or were abstinent...
during the study. All subjects gave written informed consent for participation in the study, which was approved by the Lothian Research Ethics Committee.

A method was sought to provide a convenient means of identifying the fertile period prior to ovulation, and thereby to time the administration of LNG. Unipath (Bedford, UK) had developed a technology that can be used in the home to monitor changes in urinary hormones [12]. This monitoring system comprises disposable test sticks and a hand-held monitor, which together are used to detect changes in the levels of oestrone-3-glucuronide (E3G), a urinary metabolite of oestradiol, and LH to indicate the potentially fertile days leading up to ovulation.

The monitor optically measures the intensity of the lines that form on the test sticks after sampling, and the system will delineate three levels of fertility (Low, High, and Peak Fertility) according to the optical signal changes detected. Low Fertility will be displayed from day 1 of the cycle until the hormone levels rise above the baseline levels. A change from Low to High Fertility is triggered by detection of elevated E3G levels, to concentrations typically between 20 and 30 ng/mL. The change from High to Peak Fertility is triggered by the detection of an LH surge, typically with a concentration higher than 30 IU/L. Peak fertility is displayed on the day of the LH surge and on the following day.

Each woman was studied during four cycles and was issued a monitor at the beginning of the study. Subjects were asked to use the monitor according to instructions, and familiarized themselves with the monitors by using it during a pre-study cycle to identify the days of high fertility and the day of the LH surge. They also recorded days of vaginal bleeding.

Data from the pre-study cycle was used to predict the timing of the LH surge and of the fertile phase during the study cycles and thereby to predict when treatment should be administered. The three study cycles followed immediately after the pre-study cycle. Six subjects were randomly assigned to treatment arm A and received LNG in the first study cycle and placebo in the third study cycle. The remaining six subjects were randomized to treatment arm B and received placebo in the first study cycle and LNG in the third study cycle. The second study cycle was a washout phase for all women during which time they also received placebo tablets.

The randomization list was produced using SPSS Rv.Bernouilli function such that each study number was randomly assigned to either treatment arm A or treatment arm B with the same probability, i.e. 0.5.

LNG and placebo were visually identical and were pre-packed. Each subject collected a sample of early morning urine daily from the first day of the first study cycle until and including the first day of the menstrual bleed signaling the end of the last study cycle. Samples were frozen and later assayed in batches (with all samples from one subject assayed in a single batch) for measurement of urinary LH, E3G, and pregnanediol-3-glucuronide (P3G).

Quantitative assessment of urinary LH was performed using an LH MAIAclone kit (BIOSTAT-DIAGNOSTICS, Stockport, Cheshire, UK). This method incorporates two high-affinity monoclonal antibodies into an immunoradiometric assay system and offers a working range of 1.5–200 mIU/mL. Urinary E3G was measured using a direct enzyme immunoassay (working range 0.25–32 nmol/L), while direct immunoassay was used to measure E3G levels (working range 8.36–2140 nmol/L). Intra-assay coefficients of variation were 6% for E3G, 10% for P3G, and 3% for LH [13]. Geometric means of daily replicates were divided by the respective daily creatinine concentration to correct for variations in the dilution of the urine specimen.

During study cycles 1 and 2, women were asked to take the study medication on the first day of High Fertility as identified by the monitor. However, by the third study cycle, the variation in the number of high fertile days (range 0–8 days) meant that the monitor could not be used to administer medication on LH-2 in every cycle. Therefore, we had to adopt a different method of calculating the anticipated day of the LH peak for each cycle based on the monitor information from the previous cycles (including the pre-study cycle). Hence, in the third study cycle, the medication was taken 2 days prior to the anticipated day of the LH peak. In all cycles the first tablet was taken at 1100 h and the second at 2300 h. A sample of venous blood was collected 5–7 days after treatment, stored and later assayed for progesterone using Coat-A-Cont solid-phase radioimmunoassay. The subjects kept a daily record of all vaginal bleeding experienced during the four cycles, the fertility status information displayed each day on the monitor LCD and the days on which the study medications were taken.

2.1. Statistical analysis

We calculated that a total of six subjects in each of the two treatment arms would give more than 90% power to detect a delay of menses of 5 days in 95% of cycles.

Preliminary analysis was performed to determine whether parametric tests were appropriate for analysis of the data. Outlying data points were investigated while the treatment was still blinded. The period effect and interaction between treatment and period effect were tested (two-sample t test) before progressing to testing of a treatment effect and was non-significant. Comparisons between LNG versus placebo cycles was tested by paired t test.

For the purpose of the study the following definitions based on the quantitative data were created.

A significant delay in the onset of next menses: Delay of 5 or more days from the expected onset of menses (based on the mean cycle length for the 2 placebo cycles).

The LH peak was defined as a significant rise in urinary LH concentration, with a minimum of 50% rise above the average baseline level for 4 preceding days and which remained elevated for a minimum of 3 days.

The first day of the LH peak was defined as the day of the
first significant rise (≥50% above the baseline) seen at the beginning of the LH peak.

Retrospectively predicted first day of the LH peak for the treatment cycles was the calculated mean of the first day of LH peak in the two placebo cycles.

A significant delay in the first day of the LH peak: Delay of 5 or more days from the expected first day of the LH peak in the treatment cycle (based on the mean first day of the LH peak during the 2 placebo cycles).

Luteal phase: time from the day after the first day of the urinary LH peak (LH+1) until, and including, the day before the first day of the next menses.

Follicular phase: time from the first day of the menses until the day of the first significant rise in urinary LH (LH+0) inclusive.

3. Results

A total of 48 menstrual cycles were studied—12 pre-study cycles and 36 study cycles. Data from daily urine samples were available for 34 out of the 36 study cycles. In one woman (S103) the first study cycle, which was a placebo cycle, was prolonged (41 days) as a consequence of a delay in ovulation. Her usual cycle length was 25 days, this cycle was excluded from the analysis. In a second woman (S110) there were no daily urine samples available from the washout cycle as she was abroad on holiday. Therefore, daily urine samples were only available for this subject from two study cycles (the treatment cycle and one placebo cycle).

3.1. Timing of administration of LNG

Six women took LNG in the first study cycle and six took it during the third study cycle. During the first study cycle, 10 women took the tablet (either placebo or LNG) on the first day of High Fertility as indicated by the monitor. The remaining 2 women took the tablet on the first day of the urinary LH peak because the monitor failed to identify any high fertile days prior to the LH surge.

The variation in the number of High Fertile days (0–8 days) declared by the monitor meant that the system could not be used to predict LH-2 in every cycle. Therefore, for cycle 3 we calculated the anticipated first day of the urinary LH peak from the information gathered from the pre-study cycle and study cycles 1 and 2 for each woman and instructed subjects to take the tablet 2 days before the anticipated day of the LH peak.

After completion of the study, we retrospectively calculated the predicted first day of the urinary LH peak for every treatment cycle based on the mean first day of the LH peak in the two placebo cycles. When we applied this retrospectively predicted definition to all 12 treatment cycles, the day of taking LNG ranged from 4 days before until 1 day after the first day of the anticipated LH peak. However, in reality, LNG was never taken after the first significant rise in urinary LH concentrations in any treatment cycle. The timing of the LH peak in each of the four cycles, the predicted day of the LH peak day and the timing of LNG and placebo treatment in relation to the start of the actual LH peak are shown in Table 1.

---

Table 1
Timing of LH peak, predicted day of the LH peak and timing of LNG in treatment cycles for all subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Day of the LH peak (LH &gt; 50%)</th>
<th>Predicted day of the LH peak in relation to the day of LH peak in the treatment cycle</th>
<th>Timing of LNG in relation to the predicted day of the LH peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-study cycle</td>
<td>Placebo (washout)</td>
<td>Treatment</td>
</tr>
<tr>
<td>S101</td>
<td>12</td>
<td>13</td>
<td>9</td>
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<tr>
<td>S102</td>
<td>M</td>
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</tr>
<tr>
<td>S110</td>
<td>18</td>
<td>18</td>
<td>££</td>
</tr>
</tbody>
</table>

*The woman with normal LH peak but no significant rise in pregnanediol in the luteal phase = anovulatory cycle.

*Women who had a delay of the LH peak by >5 days.

*Excluded, unusually delayed ovulation.

££ = excluded, no daily urine available.

M = LH peak not detected by the monitor.
Table 2

<table>
<thead>
<tr>
<th>Mean cycle length</th>
<th>Treatment cycles</th>
<th>Placebo cycles</th>
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<tbody>
<tr>
<td>All cycles, n = 12*</td>
<td>32.17 (SD ± 3.36)</td>
<td>26.33 (SD ± .42)</td>
</tr>
<tr>
<td>N = 4, Delay of &gt;5 daysb</td>
<td>42.75 (SD ± 8.42)</td>
<td>27.13 (SD ± 1.84)</td>
</tr>
<tr>
<td>N = 8, remaining cyclesc</td>
<td>24.88 (SD ± 2.1)</td>
<td>26.13 (SD ± 1.69)</td>
</tr>
</tbody>
</table>

*p = 0.14.
*p = 0.04.
*p = 0.12.

3.2. Cycle length

Treatment with LNG in the pre-ovulatory period significantly prolonged by 5 days or more the mean cycle length in four women (33% of the sample, Table 2). All 4 women reported vaginal spotting 2 to 3 days after taking LNG and they all had a second episode of vaginal bleeding between 9 and 16 days after the delayed LH peak. In the remaining eight women, there was no significant difference in cycle length between treatment and placebo cycles. One of this group of women, however, reported light vaginal bleeding starting a week after taking LNG, the bleeding continued until she started what she regarded as a normal menstrual period which followed a fall in urinary pregnanediol levels. Her hormone profile during the treatment cycle followed a normal pattern.

3.3. The first day of the LH peak

In the four women with long cycles, LNG appeared to abort the LH peak and a subsequent LH peak occurred 7 to 16 days later, followed by a normal rise in urinary pregnanediol. The urinary hormone profile of one woman (S110) is illustrated in Fig. 1. In the remaining eight women, LNG did not affect the timing of the LH peak when taken immediately before ovulation. Fig. 2b illustrates the hormone profile in one of these women (S101).

3.4. Length of the luteal phase

In all 12 volunteers the luteal phase was significantly shortened following treatment with LNG as compared with the placebo cycles (mean length 11.5 days [SD ± 1.8] vs. 12.9 days [SD ± 2.5] p = 0.005, Table 3).

3.5. The effect on total LH during the luteal phase

Daily urinary LH concentrations were summed from the first day of the LH peak (LH+0) up to the day before the first day of the next menses to give a value for total LH concentrations. The 8 women in whom pre-ovulatory LNG did not affect the cycle length, showed a significant (p = 0.01) decrease in total LH in the treatment cycles (18.7 U/mmol,

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Fig. 1. Daily levels of LH (●) oestrone-3-glucuronide (E3G) (△), and pregnanediol (○), in urine relative to the cycle day. (a) Placebo cycle of a woman (S110) (b) Treatment cycle of the same woman (S110) showing significantly prolonged cycle following pre-ovulatory LNG. ○, Day of taking LNG or placebo tablet; --- ---, day of the LH surge.
Fig. 2. Daily levels of LH (●), oestrone-3-glucuronide (E3G) (△), and pregnanediol (○), in urine during the treatment cycles. (a) Of the woman (S105) with no significant rise in pregnanediol following LNG. (b) Of a woman (S101) with apparently normal cycle length. •, Day of taking LNG; ◢, day of the LH surge in the placebo cycle; ——, day of the LH surge.

SD ± 8.9) as compared with the placebo cycles (27.1 U/mmol, SD ± 13.6). In contrast, among the 4 women with significantly longer cycles after taking LNG, there was no difference in the total LH secretion in the luteal phase (mean total LH = 18.8 mIU/mL, SD ± 8.3; versus mean total LH for placebo cycles of 17.8 U/mmol, SD ± 2.8; \( p = 0.81 \)).

3.6. Effect on pregnanediol in the luteal phase

The sum of daily pregnanediol concentrations in the luteal phase (from LH+1 onwards up to the day before the first day of next menses) was compared during the treatment and placebo cycles was employed to indirectly assess the function of the corpus luteum.

In one woman (S105) after taking LNG, there was no significant rise in urinary pregnanediol levels (>0.5 mmol/mol creatinine as expected in the mid-luteal phase) despite an apparently normal LH peak. The mid-luteal serum progesterone level during this treatment cycle was consistent with an anovulation (<5 nmol/L) (Fig. 2a). In the remaining 11 subjects, the total values of pregnanediol did not show a significant difference between placebo or treatment cycles.

4. Discussion

Evaluation of daily hormone concentrations confirmed that all 12 women in our study took LNG before the LH peak, and presumably ovulation. Seven women had apparently normal ovulatory cycles after taking LNG. Five of them took LNG on the day before the LH surge and one on the day of the surge. It is possible that the timing of LNG in these women was “too late” to influence an event already well underway. However, in the four women in whom the LH peak and ovulation was delayed, the LNG was taken within 3 days of the predicted LH peak. One woman did not ovulate at all despite having an LH surge 2 days after taking LNG.
It is apparent that different women respond differently to the administration of LNG. The effects observed may be related to administering LNG at a specific stage in follicular development. Even though the timing of the LNG in relationship to the onset of the LH surge did not appear to be different between women in whom ovulation was affected and those in whom it was not, it would be naïve to accept that our predicted day of the LH peak based on information gathered in two cycles was always accurate. A more detailed study employing daily serum levels of gonadotrophins and steroid hormones and ultrasound scans to correlate follicular size and maturity to the timing of the administration of LNG might provide an explanation.

The 7 women, who apparently ovulated normally, had a reduced total luteal LH and a shortened luteal phase. Basal levels of LH are essential for the normal secretory function of the corpus luteum [14]. In the mid-luteal phase, LH inhibition by the administration of GnRH antagonists consistently results in luteolysis in women as well as in nonhuman primates [15–17]. There are no direct ways of measuring whether the function of the corpus luteum is compatible with the establishment of pregnancy. Although there was no significant difference in the urinary pregnanediol levels after LNG, it is possible that the shortened luteal phase observed was a consequence of reduced total LH and may have a contraceptive effect.

If LNG acts as an emergency contraceptive only by interfering with ovulation, the expected efficacy should fall below 42% (5 of 12 women). Ho and colleagues [1] reported that LNG reduced approximately 60% of the expected number of pregnancies (estimates were based on the table of probabilities of pregnancy at different cycle days by Dixon et al. [18]). LNG fared better in the WHO study [2] with overall 85% reduction of expected number of pregnancies (the analysis of the prevented fraction was based on the modified Wilcoxon estimates of conception probabilities [19]).

Both studies reported effectiveness against estimates based on historical data. The fertile period was determined on the assumption that ovulation occurred 14 days before the next expected menses. The validity of using these estimates directly relies on the accuracy of reported menstrual cycle data. Women do not regularly keep records of their menses, and by and large the sexual intercourse responsible for requesting emergency contraception is unplanned. Reporting errors are common and the estimates can be inaccurate. In addition, other factors such as biologic variability of the day of the ovulation and the length of the luteal phase, factors affecting the probability of pregnancy unrelated to the timing of intercourse, and heterogeneity among couples in fecundability can distort the estimated number of pregnancies. In a study comparing the efficacy of the Yuzpe regimen of EC with a single dose of mifepristone [20], there were frequent discrepancies among subjects between the stage of the cycle as estimated from the date of the LMP and that suggested by circulating concentrations of progesterone. There has never been a placebo-controlled trial of EC. Thus, it is possible that the genuine effectiveness of LNG as an emergency contraceptive is less than 42%.

One woman (S103) in our study showed a delayed LH peak (on day 27) during a placebo cycle and subsequently the length of that cycle was prolonged to 41 days (her usual cycle length was 25 days). In contrast to the four women who experienced similar prolongation of the cycles after taking LNG, this woman did not report any intermenstrual vaginal bleeding. Although we excluded this cycle from our analysis, similar spontaneously occurring prolonged cycles (with delayed ovulation), can influence the results of studies into emergency contraception.

The discrepancy noted in the estimated effectiveness of LNG and the prevalence of ovulation delay or inhibition in our study may be due to mechanisms of action other than interference with ovulation. Our study was not designed to investigate the other possible mechanisms by which LNG works. However, one woman in our study reported slight vaginal bleeding after taking LNG with an apparently normal LH peak, cycle length and hormone profile. This may suggest an additional effect of LNG on the endometrium [21–24]. Nevertheless, the question remains as to whether similar alterations occur in the endometrium after taking the emergency contraceptive regimen of LNG, and whether these changes are sufficient to prevent implantation and account for the observed contraceptive efficacy of LNG. The effect of progestogens on cervical mucus and on the cervix is well documented and this is thought to be the main mechanism by which the progestogen-only pill exerts its antifertility action [25–27]. However, even if LNG has an effect on cervical mucus, which interferes with sperm penetration, that action is unlikely to prevent pregnancy when taken some 12–72 h after coitus.

The reason for using the monitor to time the administration of LNG or placebo was to avoid having to subject the volunteers to regular blood samples and ultrasound scans.

However, due to the variability in the number of high
fertile days declared prior to the LH surge, greater reliance had to be placed on calendar calculations to predict the LH surge.

In conclusion, we suggest that LNG taken immediately before ovulation acts as an emergency contraceptive by delaying or preventing ovulation. Other plausible actions of LNG including the retardation of the endometrium, interfering with sperm motility and altering cervical mucus may be important, and need to be explored further.

Acknowledgments

The authors thank Dr. Andre Ulmann, HRA Pharma, for providing us with the study medication, Unipath Ltd. for the provision of home use monitors and data collected from the monitors, Dr. Rob Elton and Ms. Dawn Everington for their assistance and advice on statistics, Mrs. Ann Mayo for helping with the recruitment of patients, and Mrs. Martha Urquhart for laboratory assays.

References


Feasibility of administering mifepristone as a once a month contraceptive pill

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Many women find the idea of a once-a-month contraceptive pill an attractive concept. Mifepristone has been shown to be effective as a contraceptive if administered in the early luteal phase. We tested the contraceptive efficacy of 200 mg of mifepristone on day luteinizing hormone (LH) + 2 in a group of 32 women who used a fertility monitor to identify the LH surge. We also recruited a control group, comprising 20 women who were trying to conceive. In this group, 12 women conceived during a total of 50 control cycles (probability of pregnancy 0.25–0.32). Women in the treatment group contributed to a total of 178 cycles and there were two pregnancies (probability of pregnancy 0.01). An LH surge was not detected in 34 cycles (19.1%). In 20 cycles (11.2%) this was due to imperfect use while 14 were monitor method failures (7.9%). Treatment with mifepristone in the early luteal phase did not disrupt the cycle length but women reported slight vaginal bleeding in 15% of the cycles. The combination of a home-use fertility monitor with once-a-month administration of mifepristone (especially if mifepristone is administered at the early luteal phase) is an acceptable contraceptive option with minimal side effects. Unfortunately, it is difficult to envisage how an easier way of defining the correct timing, which required less compliance, could be devised.

Key words: contraceptive/home use fertility monitor/LH surge/Mifepristone/once-a-month pill

Introduction

Hormonal contraception is used by almost 100 million women world-wide. However, many women are deterred from using it because of perceived risks to health such as breast cancer or side effects such as weight gain. Most of the risks and the side effects are the results of prolonged exposure to steroids and many women, in a variety of cultural settings, find the idea of a pill which they need take only once each month, an attractive concept (Rimmer et al., 1992; Glasier et al., 1999).

Progesterone is essential for the establishment and maintenance of human pregnancy. The anti-progesterone mifepristone is a synthetic 19-norsteroid, which acts by blocking the action of progesterone at the receptor level (Spitz and Bardin, 1993), and thus, has multiple potential anti-fertility actions. When administered in the early luteal phase mifepristone retards endometrial development, without disturbing the timing of menses (Swahn et al., 1988; Berthois et al., 1991; Maentausa et al., 1993). It also alters urinary contractility to a pattern more usually seen in the late luteal phase (Gemzell-Danielsson et al., 1990). In 1993 Gemzell-Danielsson and colleagues conducted a pilot study in which a single dose of 200 mg of mifepristone was given in the early luteal phase [2 days following the surge of the luteinizing hormone (LH) in urine]. Out of 124 cycles in which coitus took place during the fertile period, only one pregnancy was observed (Gemzell-Danielsson et al., 1993). There was no disruption of the timing of the subsequent menstrual bleed, although in 35% of the cycles slight vaginal bleeding was reported 2–3 days after treatment.

The main problem in developing a once-a-month contraceptive is finding a means that, both reliably and easily, identifies the start of the LH surge. Gemzell-Danielsson tried to solve this problem by using the LH sticks for home urine testing (Ovu-quick; Organon). In their study 12 out of 169 cycles were deemed to be anovulatory. However, it is not possible to determine if the LH surge truly was absent, or if the method failed to detect a surge. The woman may have read the test result wrongly or even failed to perform a test on the appropriate day.

Unipath (Bedford, UK) have developed a technology that can be used in the home to monitor changes in urinary hormones. This system comprises disposable test sticks and a hand held monitor, which together are used to detect changes in the levels of oestrone-3-glucuronide (E3G), a urinary metabolite of oestradiol, and LH, to indicate the potentially fertile days leading up to ovulation. The time from the first significant rise of LH in the urine to ovulation is reported to be around 24–48 h (Collins, 1996). The monitor thus should provide a convenient method of identifying the early luteal phase. Summary data for up to six consecutive cycles can be stored in the monitor memory and these data can be retrieved.
We investigated the contraceptive efficacy of 200 mg of mifepristone on day LH + 2 in a group of women who used this monitor to identify the LH surge.

Materials and methods
This was a single centre study in healthy female volunteers, approved by the Lothian Research Ethics Committee. All subjects gave written informed consent to participation. Fifty-two sexually active women, with regular (25–32 day) menstrual cycles were recruited from a large Family Planning Clinic in Edinburgh. If the women had a significant medical condition or if they or their partners had a history of fertility problems, they were excluded from the study.

Treatment group
Thirty-two women were recruited to the treatment group. None had been taking hormonal preparations within the 2 months prior to the start of the study and all had had at least two spontaneous menstrual periods since stopping hormonal contraception. All women underwent screening at the time of recruitment including a routine physical and gynaecological examination. A venous blood sample was taken for full blood count, serum biochemistry and liver function. The study started on day 1 of the menstrual period following screening, and lasted for up to seven consecutive menstrual cycles in which subjects took 200 mg mifepristone once per month.

Control group
The control group consisted of 20 healthy women with regular menstrual cycles who were trying to become pregnant (for less than 6 months prior to the enrolment in to the study) and hence, were not using contraception. They were provided with a monitor, which they used according to the manufacturer’s instructions. Women were advised that their chance of conception would be higher if they were to have sexual intercourse during the fertile period, identified by the monitor. The controls took part in the study until pregnancy occurred or for a maximum of six cycles if they did not conceive.

Procedure
All subjects and controls were provided with a home use hormone monitoring system (Unipath, Bedford, UK). The system comprises a hand-held monitor and disposable dual-assay urine test sticks, and is used to simultaneously detect LH and E3G levels in early morning urine. The monitor optically measures the intensity of the lines that form on the test sticks after sampling, and the system will delineate three levels of fertility (Low, High and Peak Fertility) according to the optical signal changes detected. Low fertility will be displayed by day 1 of the cycle, until the hormone levels rise above the baseline levels. A change from low to high fertility is triggered by detection of elevated E3G levels, to concentrations typically between 20 and 30 ng/ml. The change from high to peak fertility is triggered by the detection of an LH surge, typically with a concentration >30 IU/L.

Peak fertility is displayed on the day of the LH surge and on the following day. Subsequently high fertility is displayed for 1 day prior to a return to low fertility. At the start of each menses, the subjects pressed the ‘m’ button on their monitor to initiate that cycle of use, at a time suitable for testing the first urine of the day. For the rest of the month, the subjects were required to consult the monitor display each morning (3 h either side of the time when ‘m’ button was set) to determine whether they needed to perform a test that day. Beyond this 6 h time window the monitor would not accept a test. The system requests one test every day for up to a total of 10 or 20 tests, depending on the length of the woman’s cycle, and the timing of her LH surge. Embedded software within the monitor collects and analyses data from each cycle to identify and display fertility status to the user, and stores data for several months.

Mifepristone (Laboratories Exelgyn, Paris, France) was taken 2 days after the day of the first day of peak fertility (LH surge). With each cycle, subjects followed the same protocol, and were reviewed by the investigator monthly, on day LH + 2. Just before taking the 200 mg tablet of mifepristone, a venous blood sample was taken, and later assayed for progesterone. At the beginning of the study, if the LH surge was not identified by day 21 of the cycle, the subject was instructed to continue testing, but mifepristone was not given in that cycle. The subject was also advised to use barrier contraception from day 21 until the onset of the next menses. After the second pregnancy (which occurred due to a failure in detecting an LH surge), we changed this practice. We calculated the estimated day of LH surge for each month based on information from the previous cycles. If the women did not detect an LH surge either within 3 days after the anticipated day of LH surge or by day 19, a blood sample was taken for rapid serum progesterone assay. If the progesterone level was >5nmol/l and if the woman was at risk of pregnancy, mifepristone was administered.

All subjects and controls kept a menstrual record card, recording all vaginal bleeding experienced during the study and the days on which they had sexual intercourse. Subjects also marked the first day of the peak fertility as identified by the monitor and the day of taking the study medication.

If menstruation was overdue by more than one week the investigator performed a pregnancy test. Provided this was negative, the subject continued in the study and the next cycle was deemed to start with the onset of menses. Since the effect of mifepristone taken in very early pregnancy is unknown, and teratogenic effects could not be ruled out, women who would not consider terminating any pregnancy were not recruited to the treatment group.

At the end of the study, the subjects attended for a final visit, when a routine physical and gynaecological examination was performed. Full blood count, serum biochemistry and liver function were reassessed.

The following definitions were created for the purpose of the study.

Imperfect use: was defined as failure to detect an LH surge through performing the test incorrectly (e.g. dipping a test stick in urine 30 or more min before it being read by the monitor), or failing to perform tests as requested by the monitor.

Monitor method failures: were defined as failure to detect an LH surge despite performing all tests as requested.

High fertile days: days preceding the urinary LH surge as indicated by the monitor to be potentially fertile.

Peak fertile days: The first day of a significant rise in urinary LH detected by the monitor, and the following day.

The fertile period: of the cycle was defined as 3 days before until 2 days after the urinary LH surge (LH–3 to LH+2).

Exposure cycles: were cycles in which women reported having sexual intercourse at least once during the fertile period.

Statistical analysis
Cycle lengths and serum progesterone concentrations were compared by two-sample t-tests. Confidence limits for efficacy were derived from confidence limits for relative risk calculated by the Greenland and Robins method (Greenland and Robins, 1985).

Results
Table I shows the demographic characteristics of the women who took part in the study.
The women in treatment group were slightly younger (mean age 30 years) than those in the control group (mean age 32.9 years). Otherwise there were no differences between subjects and controls.

**The probability of pregnancy in the control group**

Twenty women were recruited to the control group and three withdrew before completing the study. Two withdrew from the study as they found using the system ‘too stressful’ and one withdrew because she no longer wished to plan a pregnancy. Data were collected from 50 control cycles during which 12 pregnancies occurred. Average frequency of intercourse was 1.7 episodes per week in the 39 control cycles in which the women kept a record of their sexual activity. In 37 cycles women had intercourse at least once during the fertile period (FP). In two cycles intercourse did not occur during the FP, while in 11 cycles the exposure status was unknown, as women failed to keep a record of sexual activity. Eight pregnancies occurred in the first exposure cycle.

If we assume that all 11 cycles from which information on sexual activity was lacking were exposure cycles, the probability of pregnancy was 0.25. However if those cycles were all non-exposure cycles, the probability of conception would be 0.32. Therefore among the control group the overall probability of pregnancy if sexual intercourse took place at least once during the fertile period lies between 0.25–0.32.

**Contraceptive efficacy of the method**

Thirty-two volunteers were treated with a single dose of 200 mg of mifepristone administered in the luteal phase of the cycle as their sole method of contraception between one and seven cycles. They contributed a total of 178 cycles, and in 167 cycles mifepristone was administered. Eight women withdrew from the study before completion; two women moved out of the area, three ended their relationship, two conceived during the study and one lost confidence in the method.

Two clinical pregnancies occurred in the 178 cycles studied. The first pregnancy was a true treatment failure, which occurred in a woman (para 1) who took mifepristone on day 14 (LH + 2) of her first treatment cycle. She opted for a surgical termination of pregnancy, which was performed at 8 weeks of gestation (confirmed by ultrasound scanning). In the second woman (para 3), an LH surge was not identified in her third study cycle hence she did not receive treatment with mifepristone, menses did not occur and on day 37 after her last menstrual period an ectopic pregnancy was diagnosed and treated surgically. In a third woman a biochemical pregnancy was diagnosed (serum βHCG only rising to 34 IU/l), which was spontaneously and completely aborted by day 34 of the third study cycle after taking mifepristone on day 14 (LH + 2). This woman continued in the study and completed six treatment cycles.

The mean frequency of sexual intercourse was 1.8 episodes per week in 167 treatment cycles in which sexual activity was recorded. If we assume the probability of pregnancy in the treatment group is similar to the control group (0.25–0.32), the expected number of clinical pregnancies during the 178 cycles (in which 140 were exposure cycles) studied should be between 35–48.3. The observed number was two. Therefore, the efficacy of the method is 94.3% (95% confidence interval 75.4–98.7) – 95.9% (95% CI 82.5–99.0).

When calculating the efficacy of the method, we excluded the 29 cycles during which women were not exposed to a risk of pregnancy, and the three cycles in which mifepristone was taken in the follicular phase.

**Contraceptive efficacy of luteal phase administration of mifepristone**

In 145 cycles in which mifepristone was taken in the early luteal phase (within 2 days of the urinary LH surge) 117 were exposure cycles (Table II). Exposure status was unknown in eight cycles and in 20 cycles women were not at risk of pregnancy. In the 117 exposure cycles, there was only one clinical pregnancy.

In 19 (10.7%) cycles, no LH surge was declared by the monitor but mifepristone was given as coitus had taken place during the fertile period of the cycle (calculated according to the usual cycle length and usual day of LH surge). Occurrence of ovulation was confirmed by serum progesterone of >5 nmol/l in all 19 cycles and treatment was administered prior to day 21 of the cycle in each case [between day 13–21 of the cycle, mean 16.9 (SD ± 2.1) days]. There were no pregnancies in these cycles.

If the probability of pregnancy in all exposure cycles in the study is 0.25–0.32 (the same as that of the control group), between 34–46 clinical pregnancies would be expected in the 136 ovulatory cycles in which mifepristone was taken in the luteal phase. The observed number was one. Hence, the contraceptive efficacy of luteal phase mifepristone is between 97.1% (95% CI 78.00–99.6) – 97.8% (95% CI 83.9–99.7).

**Performance of the home use hormone monitor**

In 140 treatment cycles an LH surge was identified by the monitor, which equates to 90.9% LH surge detection when calculated for perfect use cycles; and 80.5% when imperfect use cycles are also included in the total. In 127 cycles this

<table>
<thead>
<tr>
<th>Table I. Demographic data.</th>
<th>Treatment group (n = 32)</th>
<th>Control group (n = 20)</th>
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</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18–39</td>
<td>26–40</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>30 (± 5.4)</td>
<td>32.9 (± 4.5)</td>
</tr>
<tr>
<td>BMI</td>
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<tr>
<td>Range</td>
<td>19–38</td>
<td>21–29</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>23.6 (± 4.3)</td>
<td>23.8 (± 2.7)</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7 (21.9)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Non-smokers (%)</td>
<td>21 (65.6)</td>
<td>16 (80)</td>
</tr>
<tr>
<td>Ex-smokers (%)</td>
<td>4 (12.5)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Previous pregnancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+ (%)</td>
<td>19 (59.4)</td>
<td>14 (70)</td>
</tr>
<tr>
<td>Never been pregnant (%)</td>
<td>13 (40.6)</td>
<td>6 (30)</td>
</tr>
<tr>
<td>Ever abortion (%)</td>
<td>15 (46.9)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Married/Co-habiting (%)</td>
<td>28 (87.5)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Single (with a regular boy friend) (%)</td>
<td>4 (12.5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
was confirmed by a subsequent rise in serum progesterone of >5 nmol/l in the early luteal phase. This information was not available from nine cycles (blood samples lost or not collected). In the remaining four cycles serum progesterone was between 2–5 nmol/l, 1 or 2 days following the urinary LH surge as detected by the monitor. This may have been due to an early detection of the first significant rise in urinary LH. None of these five cycles were prolonged after taking mifepristone, hence it is unlikely that they were anovulatory.

There was a total of 38 (21.3%) cycles in which an LH surge was not detected. Among them, one (0.6%) was an anovulatory cycle, defined by serum progesterone not rising above 5 nmol/l in the mid-luteal phase. In three (1.7%) other cycles we administered mifepristone on day 19, before the monitor had identified an LH surge. Serum levels of progesterone (taken on the day of administering mifepristone) confirmed that in these cycles mifepristone was administered in the follicular phase. All three cycles were prolonged (43–52 days).

In the remaining 34 cycles an LH surge probably occurred (as suggested by a rise in serum progesterone of >5 nmol/l) but was not identified by the monitor. Fourteen were missed due to monitor method failure (7.9%) and 20 were a consequence of imperfect use of the system (11.2%).

**Cycle length**

Mifepristone when given in early luteal phase did not significantly affect the cycle length (P = 0.35). The mean of the usual cycle length was 28.3 days (SD ± 1.3) and during the treatment cycles it was 28.0 days (SD ± 1.9).

**Side effects**

Women kept a record of vaginal bleeding in 139 out of the total 144 cycles where mifepristone was taken on LH + 2. Mifepristone induced vaginal bleeding within 72 h in 21 cycles (15%). In a further 19 cycles, our volunteers took mifepristone in the luteal phase but the LH status was not known. In 17 of those cycles (>89%), mifepristone induced a vaginal bleed.

Serum progesterone values in blood samples taken just prior to mifepristone administration were available for 136 cycles. The mean serum progesterone value was significantly (P < 0.0001) higher in those cycles where mifepristone induced bleeding when compared to the mean value for the cycles without bleeding [21.72 (SD ± 9.04) nmol/l versus 13.33 (SD ± 6.23) nmol/l].

Two women spontaneously reported improvement of their pre-menstrual symptoms during cycles in which mifepristone was administered, while one reported worsening. In one woman hepatic alanine aminotransferase (ALT) was elevated at 103 IU/l (normal range 10–40 IU/l) at the end of the study but returned to normal within 2 months. One woman complained of diarrhoea 12 h post mifepristone in one cycle, three reported menstrual cramping within 72 h of taking mifepristone; two women reported a reduction in menstrual blood loss.

**Discussion**

A single dose of 200 mg of mifepristone administered once a month is an effective contraceptive method with an overall efficacy of 95% increasing to 97% if administered at the correct time (i.e. the early luteal phase). Thus our results are in agreement with the findings of a previous study (Gemzell-Danielsson et al., 1993).

One criticism of previous work in this field has been the lack of a suitable control group for the subjects studied. Unlike the Gemzell-Danielsson study, we were able to compare the results with a contemporaneous control group using the same methodology in the same cultural setting. In this control group, if sexual intercourse took place on a fertile day the probability of a pregnancy was 0.25–0.32. The calculated probability of pregnancy in a cohort of couples monitored during a study of natural family planning (WHO, 1983) was 0.486 if intercourse took place 3 days prior to and a day after the peak day of mucus discharge. The difference in the probability of pregnancy between our study and a variety of other published series (Table III) may be explained by the fact that we have extended our definition of the fertile period to 6 days (3 days prior to the urinary LH surge until 2 days after). Other authors (Wilcox et al., 1995) have calculated that the likelihood of conceiving during an ovulatory cycle to be 0.37 (95% confidence interval 0.31–0.48) if daily sexual intercourse took place during a 6 day fertile period (four days before and a day after ovulation). The lower frequency of intercourse in

<table>
<thead>
<tr>
<th>Table II. Treatment cycle details.</th>
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<tr>
<td>Mifepristone administered</td>
</tr>
<tr>
<td>In follicular phase</td>
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<tr>
<td>In luteal phase</td>
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<tr>
<td>Early luteal phase</td>
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<tr>
<td>LH + 2</td>
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<tr>
<td>LH + 1</td>
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<tr>
<td>LH + 0</td>
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<tr>
<td>In luteal phase (unknown LH status)</td>
</tr>
<tr>
<td>Mifepristone not given</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*a* LH surge missed, at risk of pregnancy but after day 21.

*b* Anovulatory cycle n = 1, LH surge missed and no risk of pregnancy n = 5.
our group (untimed intercourse averaging 1.7 per week) may also explain the lower probability of pregnancy.

The limiting factor in this once-a-month approach to administering anti-progesterone is the accurate detection of the LH surge. Clearly, the failure to detect accurately the LH surge has a big impact on the overall effectiveness of the method. Using laboratory assay of LH in blood or urine to identify ovulation is neither practical nor convenient for long term use in the general population. The monitor provided us with an opportunity to overcome these problems. Gemzell-Danielsson et al. (1993) reported 49% accuracy using home LH detection sticks (Gemzell-Danielsson et al., 1993). Although the monitor performed better (over 80.5% accuracy), both of these methods remain below the required standard. We studied 32 women over a total of 178 cycles. Imperfect use of the system accounted for failure to identify an LH surge in 11.8% cycles while 7.9% were due to monitor method failure. Compliance difficulties are associated with all contraceptives and non-compliance in ~12% of cycles is probably no worse than with any other method which demands action from the user, for example, compliance rates reported from oral contraceptive pill users range from 3.4–100% (Whelbe et al., 1981; Molloy et al., 1985; Hamilton and Hoogland, 1989). Although our study population consisted of women who were motivated and committed and some of them already had experience in using natural family planning methods, they found the short, inflexible testing window set on day 1 of the cycle to be particularly demanding. This is inconsistent with couples using the monitor in order to get pregnant (Bonnar et al., 1999). The prevalence of imperfect use is likely to rise in the general population compared with that typical of a research study.

During the course of the study we developed an algorithm (Figure 1) for the administration of mifepristone if an LH surge was not identified. In 19 exposure cycles (out of 28 cycles in which an LH surge was not identified) mifepristone was administered using this algorithm and there were no pregnancies. Given that the methods available to be used in real life to time the administration of mifepristone cannot be 100% accurate, such an algorithm will be essential to deal with a missed LH surge.

In our study, mis-timed administration of mifepristone led to predictable effects. When administered during the proliferative phase of the menstrual cycle, mifepristone inhibited follicular development, and delayed the mid cycle LH surge, leading to a delay in ovulation and subsequent prolongation of the menstrual cycle (Liu et al., 1987; Luukkainen et al., 1988; Swahn et al., 1988). Ovulation may occur later in that cycle, leaving women at risk of conception. In our study, when administered in the late follicular phase (in error) in three cycles, mifepristone prolonged the cycle length (43–52 days). The women were advised to use condoms for the remainder of that cycle and none of the three cycles resulted in pregnancy.

Administration of mifepristone in the mid or late luteal phase induces a bleed within a few days of treatment, which may or may not be followed by a second bleed at the time of expected menstruation (Shoupe et al., 1987; Swahn et al., 1988). In our study, in 17 out of the 19 cycles where mifepristone was taken after ovulation (the LH status unavailable and probably later than on LH + 2), inter-menstrual vaginal bleeding occurred (89.5%). Moreover, there was an increased risk of bleeding seen in those women who may have taken mifepristone slightly later in the LH + 2 window. The mean serum progesterone concentration was significantly higher in those women who had bleeding after taking mifepristone within LH + 2, when compared with those who did not. The higher serum progesterone value in some on LH + 2, could be due to a delayed identification of the first significant rise in urinary LH, or because of a more rapid increase in serum progesterone due to early ovulation. Nevertheless, in our group of women, in all cycles where mifepristone induced a vaginal bleed, a second bleed occurred at the time of the expected menses. Therefore, while the bleeding may have been inconvenient, it did not jeopardise efficacy or continued use of the method. There was less inter-menstrual bleeding (15% of the cycles) reported in our study when mifepristone was taken within LH + 2, less than half of that reported by Gemzell-Danielsson et al. (32%) (Gemzell-Danielsson et al., 1993). This is possibly due to the fact that the majority of women in our study received mifepristone at the correct time. In their study, in 51% of the cycles, mifepristone was taken between 3 and 5 days after the LH surge.

In conclusion, the use of the combination of home use

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**Table III. Probability of clinical pregnancy.**

<table>
<thead>
<tr>
<th></th>
<th>No. of exposure cycles</th>
<th>No. of pregnancies</th>
<th>Probability of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilcox et al., 1995&lt;sup&gt;a&lt;/sup&gt;</td>
<td>129</td>
<td>34</td>
<td>0.26</td>
</tr>
<tr>
<td>Our control group&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37-48</td>
<td>12</td>
<td>0.25-0.32</td>
</tr>
<tr>
<td>Our treatment group&lt;sup&gt;b&lt;/sup&gt; (monitor + mifepristone)</td>
<td>140-151</td>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>Our treatment group&lt;sup&gt;b&lt;/sup&gt; (mifepristone in luteal phase)</td>
<td>136-143</td>
<td>1</td>
<td>0.007</td>
</tr>
<tr>
<td>Gemzell-Danielsson et al., 1990&lt;sup&gt;b&lt;/sup&gt;</td>
<td>124</td>
<td>1</td>
<td>0.003</td>
</tr>
<tr>
<td>WHO study&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72</td>
<td>35</td>
<td>0.48</td>
</tr>
</tbody>
</table>

<sup>a</sup>The length of the fertile period defined as 4 days.
<sup>b</sup>The length of the fertile period defined as 6 days.

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**Figure 1. Algorithm for administering mifepristone when the LH surge is not identified.**
fertility monitor with once-a-month administration of mifepristone (especially if mifepristone is administered at the early luteal phase) is an attractive contraceptive option with minimal side effects. However, to be an effective contraceptive method, the women have to be committed to using a device, which identifies the LH surge, in order that the pill can be taken at the correct time in the cycle. Whilst this regimen may be acceptable to motivated women, it may be regarded as too complicated for others to adopt on a routine basis. There was evidence of such non-compliance in this study, with 11.2% of LH surges being missed as a consequence of imperfect use of the monitor. Unfortunately, it is difficult to envisage how an easier way of defining the correct timing, which obligated less compliance, could be devised.

Acknowledgements

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References


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Noncompliance among a group of women using a novel method of contraception


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Objective: To compare the incidence of noncompliance measured objectively by a home-use fertility monitor with the traditional self-reported incidence of compliance in a study of a new method of contraception.

Design: Prospective cohort study.

Setting: A large family planning clinic in Edinburgh.

Patient(s): Thirty-two healthy women who took part in a trial assessing the efficacy of a novel method of contraception involving accurately timed administration of a single dose of mifepristone.

Intervention(s): Mifepristone was administered orally and a blood sample was collected on the same day.

Main Outcome Measure(s): Percentage of missed tests detected by the monitor against the self-reported percentage during the critical period.

Result(s): Women failed to perform 24.2% (95% confidence interval, 16.5-31.5) of the tests in the 162 cycles analyzed. They missed tests at an absolutely vital time for contraceptive efficacy in 42% of cycles according to the monitor while admitting to missing tests in 14.8%. Poor compliance was associated with younger women, those who discontinued the study before completion, and cycles in which women were not relying on the contraceptive method.

Conclusion(s): The use of microelectronic monitoring systems may improve our understanding of the extent of patient noncompliance, providing objective information that no other monitoring technique can produce. This understanding provides the opportunity to make the optimum use of potentially effective treatments while validating research evidence. (Fertil Steril® 2001;76:1196-1201. ©2001 by American Society for Reproductive Medicine.)

Key Words: Compliance, contraceptive research, home-use fertility monitor, mifepristone.

Since the first woman on earth chose to contradict the instructions of her Provider in the Garden of Eden, expecting her descendants to comply perfectly with a contraceptive regimen may seem rather unrealistic. However, noncompliance with a particular contraceptive method is linked with an increased risk of unintended pregnancy (1). Conventionally, it has been assumed that the users of contraception are highly motivated because the consequence of noncompliance—pregnancy—is so obvious, and so significant. Studies involving organ transplant recipients have shown, however, that no consequence of poor compliance is severe enough—not even the rejection of a transplanted kidney—for all patients to reliably follow their prescribed regimen (2).

Poor compliance—in both clinical practice and research—is associated with a number of factors. Though several patient characteristics (e.g., age, education, socioeconomic background) (3-5), characteristics of the treatment regimen (e.g., frequency of dosing, side effects) (6-8), and outcome characteristics (e.g., treatment of incurable or terminal illnesses) (9) have been associated with nonadherent behavior, there are no reliable and universally applicable predictors of noncompliance. In addition, there is no gold-standard measurement for patient compliance (10).

Prevalence of noncompliance ranges from 0-96.6% in oral contraceptive pill users (11-13). Although extremely common, there is a dearth of information available on patient noncompliance with the use of different contraceptive methods. What data exist commonly come from self-reporting. When undetected, poor compliance can invalidate results of efficacy.
studies (14, 15), yet it is commonly overlooked in clinical research (16).

We observed behavior of 32 women who took part in a trial assessing the efficacy of a novel method of contraception (17). The women took 200 mg of mifepristone 2 days after the midcycle LH surge (measured in urine) as a once-a-month contraceptive pill. Mistimed administration of mifepristone can disrupt the menstrual cycle and can also leave the women at risk of conception. A home use fertility monitor that could store data on daily testing events was used to time the administration of mifepristone. Although it made minimal demands on the users, the method provided objective, long-term data on their routines. Because investigators saw participants once each cycle throughout the study, we were able to compare the results of this method with the traditional self-reported incidence of compliance.

MATERIALS AND METHODS

Data were collected during a study assessing the feasibility of administering mifepristone as a once-a-month contraceptive pill, and detailed study methodology has been reported elsewhere (17). Thirty-two sexually active women (age range, 18–39 years) were enrolled from a large family-planning clinic in Edinburgh. (The mean body mass index was 24 ± 4.3, and 66% were nonsmokers.) All subjects gave written informed consent to participate, and the Lothian Research Ethics Committee approved the study. Data were collected from a total of 178 cycles, with each subject contributing between one and eight cycles. It was not possible to retrieve compliance data from the monitors in 28 of the study cycles because of infrequent downloading from the monitor and lost or broken devices.

The study started on day 1 of the menstrual period after screening and lasted for up to eight consecutive menstrual cycles. The subjects were relying on once-a-month treatment with mifepristone as their sole method of contraception during 150 of the cycles analyzed. In addition, it was possible to retrieve data from a further 12 cycles during the study suspension period, during which women did not rely on our method for contraception but only used the monitor. During the cycles in which mifepristone was administered, the timing of mifepristone depended on detection of the LH surge, which in turn depended on compliance with daily urine testing.

Procedure

All subjects were provided with a home use fertility monitor (Unipath, Bedford, UK). The system comprises a hand-held monitor and disposable dual-assay urine test sticks and is used to detect simultaneously LH and estrone-3-glucuronide levels in early-morning urine. The system delineates three levels of fertility (low, high, and peak fertility) according to the optical signal changes detected. At the start of each menses, the subjects pressed the m button on their monitors to initiate that cycle of use at a time suitable for testing the first urine of the day.

For the rest of the month, the subjects were required to consult the monitor display each morning (3 hours on either side of the time when the m button was set) to determine whether they needed to perform a test that day. Without this 6-hour time window, the system would not accept a test. The monitor requests one test every day for up to a total of 10 or 20 tests, depending on the length of the woman’s cycle and the timing of her LH surge. Embedded software within the monitor collects, analyzes, and stores data for several months.

The correct way to use the monitor (including the importance of the testing window) was demonstrated to all subjects at the time of recruitment, and written information was given. Subjects were advised to contact the investigator immediately if they were not able to perform a test during a critical period. The investigator was available by telephone 7 days a week.

In each cycle, subjects were reviewed by the investigator monthly, on day LH + 2. A sample of venous blood was collected before taking the tablet of 200 mg mifepristone and was later used for the analysis of progesterone. The information collected by the monitor was later analyzed using special computer software. The estimated day of LH peak for each month was calculated based on information from all the previous cycles monitored. If an LH surge was not detected either within 3 days after the anticipated day of LH peak or by day 19 of the cycle, a blood sample was taken for rapid serum progesterone assay, and the information from the monitor was downloaded. Mifepristone was administered only if the woman was at risk of pregnancy (i.e., had been sexually active) and if the progesterone level was >5 nmol/L.

All subjects kept a menstrual record card, recording all vaginal bleeding experienced during the study and the days on which they had sexual intercourse. They also marked the day of the LH surge as identified by the device and the day of taking the study medication.

The following definitions were created for the purpose of the study. The period of time between the calculated earliest day an LH peak was likely to occur (based on the usual cycle length and the day of the LH surge in previous cycles) and the day of the actual LH surge in each cycle was defined as the critical period for each patient. The fertile period of the cycle was defined as 3 days before until 2 days after the urinary LH surge (LH−3 to LH+2). Exposure cycles were cycles in which women reported having sexual intercourse at least once during the fertile period. Noncompliance was defined as a urine test that was requested by the monitor but missed.

Statistical Methods

Some of the compliance data were summarized for descriptive purposes using numbers of cycles or tests as de-
nominators. However, statistical inference was carried out on data aggregated to patient level, to take account of possible heterogeneity in behavior among patients that might have invalidated analyses using data from individual cycles or tests. Thus, percentages of tests missed were calculated for each patient, and these were tested for association with demographic data using Pearson correlations. Testing was done on data aggregated to patient level. Thus, for example, correlating each patient's age with the percentage compliance over all her cycles tested the association between age and compliance. Similarly, percentages of tests missed at different stages of the cycle, and when on and off the treatment regime, were compared by paired t-tests. A two-sample t-test was also used to compare the percentage of tests missed in patients who dropped out and those who did not.

RESULTS

One hundred sixty-two cycles were analyzed, of which 150 were study cycles. Data collected during the 12 cycles during which subjects were using a barrier method throughout the cycle and did not receive mifepristone were analyzed separately.

In total, 2,013 tests were requested by the monitor during the 162 cycles analyzed (12.4 tests per cycle, 95% confidence interval (CI), 11.8–13.0), and 494 were missed (24.2%; 95% CI, 16.5–31.5). On average, three tests (95% CI, 2.4–3.6) per cycle were missed.

Compliance During the Intention-to-Treat Cycles

During the 150 cycles in which women were relying on the study method as their only contraception, a total of 1,816 tests was requested by the monitor, and 411 tests were missed (22.6%; 95% CI, 15.2–30.1).

Compliance Before and After Identifying the Peak

In the days before the women knew an LH surge had occurred, 23% of the requested tests were missed (95% CI, 16–30; 260 of 1,160). Women were not more likely to miss tests before the LH surge than after it (t = 0.57, NS).

Concordance of Monitor Data With the Self-Reported Data

In 68 cycles (42%), women failed to test at all on a day of the cycle, which was critical to the accurate detection of an LH surge. In 27 of these cycles, the monitor did not detect an LH peak. In the remaining 41 cycles, despite critical tests being missed, the monitor did detect an LH peak. Women admitted to not performing tests in 24 cycles (14.8%). In the other 44 cycles, women did not report missed tests and only admitted to it once the investigator showed the downloaded monitor data to them.

Some women actively fabricated the information that they reported to the investigator. In one of these cycles, when contacted, a woman declared detecting an LH peak on a day (day 13 of the cycle) when she had not performed a test at all. Another woman performed the tests correctly and identified an LH peak on day 13 but forgot to inform the investigator and failed to obtain the mifepristone tablet as per protocol. When contacted on day 19 of the same cycle, she claimed that the monitor had not detected an LH peak.

Compliance During the Critical Days

Noncompliance with urine testing as monitored by the system was significantly lower during the critical period (15.6%; 95% CI, 9.5–21.7) when compared with during the noncritical days (27.5%; 95% CI, 17.9–37; paired t-test, t = 3.64, P < .01). The self-reported percentage of missed tests during the critical period was 2.7% (95% CI, 1.0–4.3), which was significantly lower than that detected by the monitor (t = 4.48, P < .001).

Compliance During the Study Suspension Interval

After a pregnancy that occurred because of a failure in detecting a LH peak, the study was suspended for a month. However, some women continued to use the monitor only, while using a barrier method for contraception. Therefore, it was possible to retrieve data from the monitors for 12 cycles in which the women were not using our method as their contraceptive.

In six of these 12 cycles, the monitor was not able to identify an LH peak because of its imperfect use. During this period, a significantly high percentage of tests (41.2%; 95% CI, 22.4–60.0) were missed when compared with the study cycles (22.6%; 95% CI, 15.2–30.1; t = 2.9, P = .015).

Compliance and Sexual Activity

There was no correlation between the frequency of sexual intercourse per cycle and the number of missed tests per cycle (correlation coefficient was 0.01). The percentage of missed tests were compared between the exposure and non-exposure cycles in 12 women who had at least one exposed and one unexposed cycle. There was no significant difference (paired t-test, t = 0.06, NS; 95% CI for differences between exposed and unexposed, −8.3 to +7.8) in compliance during exposed and unexposed cycles.

Demographic Features and Compliance

Age was negatively correlated with the percentage of missed tests—younger women missed more tests (r = −0.36, P < .05). There was no apparent relationship between compliance with the method and the number of pregnancies, number of previous abortions, or number of living children.

Compliance Among Dropouts

We analyzed cycle data from the seven women who discontinued the study before completion. They missed a significantly higher percentage of tests (44.4%) when compared with the other 25 women who completed the study.
Information collected from the monitor and the diary card from a woman with a 31-day cycle in which the LH surge was identified. Mifepristone was administered on day 20 (LH+2). (A), Information downloaded from the monitor. Level of fertility displayed to the woman on each day of the cycle: open block, low fertility; partially closed block, high fertility; closed block with circle, peak fertility. Data on testing events appears as a narrower bar underneath: open block, test performed; closed block, test missed. (B), Data recorded on the diary card by the woman (self-report). B, days of vaginal bleeding; check mark, tests done during the critical period; X, days in which sexual intercourse occurred; T, day when mifepristone was taken. (C), Downloaded information on signal levels of LH and estrone-3-glucuronide (E3G) levels. Closed circle, LH; open square, E3G; M, missed tests. Note that on several days (comparing panels A and B), the woman reported performing the test (check mark) when the monitor showed that she did not (M).

(A) Panel I

(B) Panel II

(C) Panel III

Day of peak fertility

E3G signal (%)

Cycle day

(17.4%; 95% CI of the differences between the two percentages, 10.6–43.3; t = 3.39, P=.002). The dropouts missed tests on a critical day in 16 of the 22 cycles, although they admitted to missing tests in only four. Therefore, the self-reported incidence of noncompliance during the critical period was 18.1%, whereas the monitor-detected incidence was 72.7%.

DISCUSSION

The home use fertility monitor offered us an important methodological advance in providing reliable data on the incidence and the magnitude of noncompliance with the contraceptive method studied and on information about exactly when the imperfect use occurred. We sought to achieve significantly high compliance rates with employing this monitoring system in a contraceptive regimen, which appeared to be acceptable to women (18, 19). Women were counseled at the start of the study regarding the importance of performing urine tests accurately to identify the time to administer mifepristone. They were aware that if mifepristone was not taken, they would be at risk of pregnancy.

Because the teratogenic effects of mifepristone are not known, women considering participation in the study were advised that if pregnancy occurred, they should consider termination. Despite this, the information collected from the monitor demonstrates that women failed to perform 24.2% of the tests in the 162 cycles analyzed. Moreover, in 42% of the cycles, women missed tests during a day that an LH surge was likely to have occurred, in other words, at an absolutely vital time for contraceptive efficacy.

Although noncompliance is common, by inviting only a selected group of women who appeared to be motivated and committed, we sought to exclude those who might be poor compliers. Interestingly, the medical profession is reportedly poor in identifying noncompliers (20). Users of our method were reasonably young (mean age, 30 years) and were healthy. The serious outcome of noncompliance (pregnancy) was rare. Women may have found the daily requirement for urine testing, using the monitor on a long-term basis, to be complicated or inconvenient. Side effects of the method (although fewer than in most other methods available) may not have been acceptable to them, or they may not have had faith in the effectiveness of the method although this seems unlikely because they did not revert to condoms. Considering these odds, it is not difficult to infer why compliance problems were frequent.

Common sense suggests that with increasing experience, the women may trivialize the need for testing on days during which an LH surge was unlikely to have occurred. All women were made aware of the critical period for testing urine at the start of the study, and as expected, women missed fewer tests during this period. Incomplete understanding of how to be compliant with a regimen has been suggested as a common reason for poor compliance (21). Women in our study were given detailed verbal and written information on using the monitor at the start of the study. The importance of accurate use of the device according to manufacturer's guidelines was stressed at each monthly visit. Because there was no significant difference in the percentage of missed tests between the first study cycle and the last study cycle, it is difficult to support the suggestion that in the earlier cycles, women were less confident about how to use the method or more confident in their ability to guess the correct time for testing (22).

There was a highly significant difference between the patient self-reported percentage of missed tests (2.7%) and that detected by the monitor (15.6%) during the critical period. Figures 1 and 2 illustrate this discrepancy during two cycles contributed.
by one woman. This discrepancy was even greater among the seven women who discontinued the study before completion and is consistent with results of previous studies that reported significant overestimation of compliant behavior with self-regulation (23). Patients tend to tell doctors what they think the doctors wish to hear. If they assume that doctors perceive patient nonadherence as a judgmental disappointment, they may feel guilty and fail to report noncompliance.

Although older women tended to miss fewer tests, the other demographic characteristics such as parity, previous abortions, number of living children, and educational background did not correlate with the number of missed tests. Neither did frequency of sexual activity or exposure to the risk of pregnancy correlate with the missed tests. However, because the information about sexual activity was only collected by self-reporting, this association should be accepted with caution.

Most investigators work on the assumption that a patient who complies with one aspect of a clinical protocol (e.g., attending the clinic as directed) also adheres to all other aspects of the study, though this might not always be the case. The monitor supplied data on daily testing of urine, which was only one part of the contraceptive method. We cannot infer from those data how well the women in our study complied with the rest of the protocol, such as recording daily events (sexual intercourse, vaginal bleeding). With no means of testing this, for validity of our results, we had to depend on our volunteers being truthful. Therefore, in the future, we have no other option but to work toward forming a true therapeutic alliance with our volunteers and to come to an agreement with our patients rather than to impose a prescription or a protocol upon them.

In conclusion, the use of microelectronics monitoring systems such as the home use fertility monitor may improve our understanding of the extent of the problem of noncompliance, providing precise objective information that no other monitoring technique can produce. This understanding will empower us as health care providers to adopt a no-fault approach to behavior relating to noncompliance and establish “a tailored consensual regimen” with the user that she is able to adhere to (24). This provides the opportunity to make the optimum use of potentially effective treatments and legitimate research evidence. Perhaps a small price to pay for such a return?

Acknowledgments: The authors thank Unipath Ltd. for the provision of monitors and data collected from the monitors, Rob Elton, Ph.D., for his assistance and advice on statistics, Mrs. Ann Mayo, S.R.N., for helping with the recruitment of patients, and Mrs. Martha Urquhart for laboratory assays.

References
Mifepristone-Induced Vaginal Bleeding Is Associated with Increased Immunostaining for Cyclooxygenase-2 and Decrease in Prostaglandin Dehydrogenase in Luteal Phase Endometrium

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The mechanism of mifepristone-induced vaginal bleeding and endometrial shedding was investigated in 13 women who took 200 mg mifepristone in the midluteal phase on d 8 after the onset of the urinary LH surge (LH+8). Endometrial biopsies were collected, 6–24 h after mifepristone (group 1, n = 7) or 36–48 h after mifepristone (group 2, n = 6), and compared with those from a control group in the midluteal phase (n = 7). All women reported vaginal bleeding commencing 36–48 h after taking mifepristone. Treatment with mifepristone significantly reduced serum progesterone levels in all women, when compared with the controls (13.2 μM vs. 34.8 μM, P = 0.001). After mifepristone, a significant increase in cyclooxygenase-2 immunoreactivity was apparent at 36–48 h (P = 0.0018), whereas prostaglandin 15 dehydrogenase enzyme-positive immunostaining declined, to be virtually absent by 36–48 h in both glands and in stroma (P < 0.05). There was no change in intensity or distribution of staining for steroid receptors after mifepristone. The changes in immunostaining for cyclooxygenase-2 and prostaglandin 15 dehydrogenase strongly support the hypothesis that an increase in the local concentration of prostaglandins in the endometrium is involved in the mechanism of bleeding induced by mifepristone in the luteal phase. (J Clin Endocrinol Metab 87: 5229–5234, 2002)

HUMAN ENDOMETRIUM is a target organ for the ovarian steroid hormones estradiol and progesterone. One of the fundamental roles of progesterone is the differentiation of an estrogen-primed endometrium (1). The endometrial receptivity that permits successful implantation depends on timed and regulated synthesis and secretion of a specific set of progesterone-induced proteins in an estrogen-primed endometrium. If a pregnancy fails to occur, the corpus luteum regresses, with a subsequent fall in progesterone levels. There is now compelling evidence that a period of exposure of the estrogen-primed endometrium to progesterone followed by a withdrawal of progesterone are the hormonal prerequisites for menstruation (2). The characteristics of the endometrial changes (including the extensive changes observed in the endometrial vasculature) associated with the withdrawal of progesterone and menstrual bleeding suggest an involvement of vasoactive local mediators. The evidence that prostaglandin (PG) activity in the endometrium is modulated by progesterone, and the widely recognized vasoactive properties of PGs, make them prime candidates for mediators of progesterone action on the endometrium (3).

PGs are synthesized from arachidonic acid (AA); thus, the liberation of AA from precursors (present as membrane-bound phospholipids) by the action of phospholipase A₂ (PLA₂) is one of the first steps (and a rate-limiting step) in PG synthesis. AA is then converted to prostanoids (including PGE₂, PFP₂x, and PGL₂) by the actions of cyclooxygenase (COX). In the endometrium, PGs are not stored but are immediately synthesized and released and metabolized to inactive metabolites by PG 15 dehydrogenase (PGDH) enzyme.

The antiprogestrone compound mifepristone (RU 486; Roussel Uclaf, Paris, France) is a synthetic 19-norsteroid with a specific high affinity for binding to the progesterone receptor (PR). It blocks the biological effects of progesterone by binding with high affinity to the PR (4). In the midluteal phase, mifepristone, at a single dose of 50–800 mg, induces menstrual bleeding within 72 h (5, 6). Luteolysis was incomplete in two thirds of the subjects, and they experienced a further episode of vaginal bleeding at the expected time of menses. In the remainder, there was complete luteolysis, with only one episode of bleeding. Thus, the vaginal bleeding observed after mifepristone, without a decrease in the circulating progesterone values, seems to be attributable to a direct effect on the endometrium. It has been suggested that the menstrual bleeding induced by mifepristone in midluteal phase is attributable to a direct effect on endometrial vessels (7). After treatment with 50 mg mifepristone in the midluteal phase (d 20–23), there is a significant reduction in the capillary luminal area and diameter associated with degenerative changes in the endothelial cells, which preceded the menstrual shedding. These changes do not always accompany regressive changes in the adjacent stroma. This effect of mifepristone on the endometrium (at a time when the en-
The endometrial effects of antigestogens given in the early-luteal phase have been extensively investigated (8–11). In the early-luteal phase, mifepristone inhibits progesterone-induced down-regulation of PR and estrogen receptors (ERs), while antagonizing the progesterone action on endometrial markers such as PGDH, which are known to be progesterone-dependent (12, 13). Moreover, PGDH has been postulated as a useful marker of the closure of the implantation window, and the effect of midluteal administration on Singh markers might add to our current understanding of potential contraceptive actions of mifepristone.

Our study investigated the mechanism of mifepristone-induced vaginal bleeding in the endometrium from 16 healthy women with regular cycles. The endometrial biopsies were performed between 0 (control) and 6-48 h after midluteal phase administration of a single dose of 200 mg mifepristone. We examined the expression and the distribution of sex steroid receptors in the endometrium and the expression of PGDH, and inducible PG synthesizing enzyme COX-2. Alterations in the expression of such progesterone-dependent proteins may widen our understanding of the mechanism by which mifepristone induces endometrial bleeding in the midluteal phase.

**Subjects and Methods**

**Subjects**

Twenty healthy women with regular cycles (25- to 30-d duration; mean age, 34 yr; range, 26–45 yr) were recruited into a randomized, single-center study with mifepristone. Women were either using a reliable nonhormonal method of contraception or were abstinent. All women underwent a comprehensive screening procedure before commencing the study. This consisted of a full medical history and routine physical and gynecological examination, together with measurement of blood pressure, pulse, height, and weight. In addition, a venous blood sample was taken for full blood count, serum biochemistry, and liver function. These blood tests were repeated at the end of the study. All women kept a menstrual diary card and recorded all vaginal bleeding experienced during the study period and in the following cycle, and the day in which they identified an LH surge using urinary dipsticks.

We also studied the endometrial samples from four women taking part in a separate study, which evaluated the secretary endometrium. Those women also used the same type of urinary dipsticks to identify the urinary LH surge, and the biopsies were collected 7 or 8 d after the first day of the urinary LH surge. Because these women did not receive any treatment, the four biopsies were included in our control group.

Therefore, the total number of biopsies analyzed in the control group was seven.

Lothian Research Ethics Committee (Institutional Review Board) approved the study, and informed written consent was obtained from each woman.

**Study design**

The women were monitored over two consecutive cycles: a treatment cycle, and a follow-up cycle. The women were allocated, at random, to 1 of 5 groups, depending on the timing of the biopsy, i.e. 6, 24, 36, or 48 h after a 200-mg mifepristone treatment on d 8 after onset of urinary LH peak (LH + 8). The control group had a biopsy but no treatment. Each of the 20 women in the study were allocated to the next consecutive study number in the randomization list before commencing the study. The randomization list was produced using the Statistical Package for Social Scientists (SPSS, Inc., Chicago, IL), such that each study number was randomly assigned to 1 of 5 groups in the study. The list was balanced after each block of 5.

Women used detection kits to detect the LH surge in a first sample of urine. On the occasion when the endometrial biopsy was taken, a blood sample was also collected for serum progesterone measurement by RIA.

Four women were subsequently withdrawn from the study: 1 because of previously undiagnosed cervical stenosis, which made endometrial biopsy difficult; in 1 woman, RIA could not confirm the self-detected LH peak; and the endometrial samples were inadequate for analysis in 2 other women. Therefore, we analyzed the endometrial samples in 13 women after taking mifepristone in the midluteal phase (n = 3, at 6 h; n = 4, at 24 h; n = 3, at 36 h; n = 3, at 48 h after mifepristone). Three samples from our original control group plus the above mentioned 4 additional samples from a separate study made a total number of 7 in the control group.

**Detection of the urinary LH peak**

The timing of the urinary LH surge was detected by the subjects themselves, using a commercially available LH detection kit (Oviquick; Unipath, Bedford, UK), which they used according to the manufacturer's instructions. The self-detected urinary LH peak was subsequently confirmed by RIA (MAI Aclone Kit; Biostat-Diagnostics, Stockport, Cheshire, UK).

**Serum progesterone**

A blood sample was collected immediately before the endometrial biopsy in all women, stored, and later assayed for progesterone. Serum progesterone measurements were done by using the Coat-A-Count progesterone procedure (solid-phase RIA; Diagnostic Products (UK) Ltd., Glyn Rhonwy, Llanberis, Caernarfon, Gwyedd, North Wales, UK).

**Endometrial biopsies**

Endometrial biopsies were obtained using a Pipelle endometrial sampling device (Prodimed, Neuly-en-Thuile, France) and were fixed immediately in 4% paraformaldehyde for 24 h, routinely processed, and embedded in paraffin, and sections were cut to 5-μm thickness. All tissue samples were labeled with a code number for anonymity; and, except for this number, the mounted sections did not contain any other information.

**Immunohistochemistry**

Immunohistochemical staining was performed for immunolocalization of: 1) PR, with a 1:40 dilution of mouse monoclonal antihuman PR antibody (0.88 μg/ml protein; Novocastra Laboratories, Newcastle upon Tyne, UK); 2) ER, with a 1:400 dilution of mouse monoclonal antihuman ER antibody ERID8 (0.58 μg/ml protein; DAKO Corp. Laboratories, High Wycombe, UK); 3) androgen receptor (AR), with a 1:480 dilution of monoclonal mouse antihuman AR antibody (F-39; BioGenex Laboratories, Inc., antibody; A Meranini Diagnostics, Berkshire, UK); 4) PGDH, with a 1:2000 dilution of rabbit polyclonal antibody (Dr. H. H. Tai, University of Kentucky, Lexington, KY); and 5) COX-2, with a 1:600 dilution of goat polyclonal antihuman COX-2 antibody (0.3 μg/ml protein; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

**Immunohistochemistry procedures**

All protocols were optimized to determine the correct conditions for maximum specific staining, and all sections used as negative controls (where the primary antibody was replaced with nonimmune IgG of the same species and concentration) did not show immunostaining. Each immunostaining procedure was performed in a single run. The immunohistochemical technique used was as follows:

Five-micrometer paraffin sections were dewaxed in Histoclear (National Diagnostics, Yorkshire, UK) and rehydrated in descending concentrations of ethanol to dH2O. After a 10-min wash in 0.01 M PBS (pH 7.4–7.6; PBS tablets, Sigma, Dorset, UK), antigen retrieval was carried out as follows: Sections to be stained for PgR and ER were microwaved for 10 min in 0.01 M sodium citrate buffer (pH 6) while those for AR and COX-2 were pressure-cooked in 0.01 M sodium citrate (pH 6) for 5 min and 2 min, respectively (Tefal, Nottingham, UK). An antigen retrieval
step was not required to expose the PGDH epitope. Sections were washed in PBS, and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in distilled water for 10 min (PR, ER, AR, PGDH) or 3% hydrogen peroxide in methanol for 30 min (COX-2). After a 10-min wash in PBS, an endogenous biotin blocking step was carried out for AR and COX-2, where sections were incubated sequentially in avidin then biotin for 15 min each at room temperature (RT) (Vector Laboratories, Inc., Peterborough, UK). After a further 10-min wash, sections were incubated in normal horse serum (Vector Laboratories, Inc.) for PR, ER, and AR or in 20% normal goat serum for PGDH, and in 20% normal rabbit serum for COX-2 (Diagnostics Scotland, Edinburgh, UK) for all 20 min at RT. The primary antibody was then added at the dilutions stated above. Sections were incubated for 60 min at 37°C for PR, ER, and COX-2 and overnight at 4°C for AR and PGDH. After washing in PBS with Tween 20, biotinylated horse antimouse antibody, followed by an avidin biotin horseradish peroxidase complex (ABC; Vectorstain Elite; Vector Laboratories, Inc.) was added for 60 min for ER and AR, and 30 min for PR at RT. Biotinylated goat antirabbit and horse antigoat (Vector Laboratories, Inc.) were added for PGDH and COX-2, respectively, for 30 min, followed by the ABC complex for 60 min (PGDH) and 20 min (COX-2) at RT. Staining was visualized by incubation in 3,3' Diaminobenzidine (DAB; DAKO Corp. Laboratories). Sections were then counterstained using Harris' Hematoxylin (Pioneer Research Chemicals Ltd., Essex, UK), dehydrated, and mounted in Pertex (Cellpath, Hemel Hempstead, UK).

Scoring and immunohistochemistry analysis

We employed a semiquantitative subjective scoring system to evaluate the intensity and the localization of immunoreactivity in entire tissue sections. Previously, we have reported that the immunostaining patterns in endometrial sections measured by the subjective semiquantitative scoring showed an almost perfect correlation with that measured objectively by computerized image analysis (14). Therefore, the less-time-consuming, semiquantitative scoring system provides a valid score suitable for graphical presentation.

Two independent observers, using light microscopy, visually assessed all coded sections. The two separate scores were then compared to obtain a more objective final score for each section. Once the final score had been agreed for all sections in the five-immunostaining runs, the code was broken. Afterwards, the final immunostaining scores were analyzed by the respective groups.

The immunostaining intensity of the steroid receptors (PR, ER, and AR) were scored using a four-point scoring scale, where the intensity of staining was assigned as 0 = none, 1 = weak, 2 = distinct, and 3 = strong. However, the staining intensity of PGDH and COX-2 showed a narrow range; and therefore, we adapted a three-point scoring scale, where the score of zero = absence of immunoreactivity, 1 = faint immunoreactivity; and 2 = strong immunoreactivity.

Statistical analysis

Originally, the sample size was determined to include 5 women in each of 4 groups at 6, 24, 36, and 48 h after mifepristone. However, because of a number of reasons listed above, only 13 biopsies after treatment were available. Because mifepristone induced bleeding by 36–48 h in all women, a preliminary analysis was performed to determine the appropriate statistical test for analysis of the data. The mean staining intensity scores between 6- and 24-h groups and between 36- and 48-h groups showed no significant difference; hence, the 13 samples after treatment with mifepristone were analyzed in 2 groups: group 1 (6–24 h after mifepristone, n = 7), and group 2 (36–48 h after mifepristone, n = 6). Comparisons between these 2 groups were tested by nonparametric Kruskal-Wallis ANOVA test and the Dunn's multiple-comparisons test because they were discontinuous data sets.

Results

All women reported vaginal bleeding commencing 36–48 h after taking mifepristone. Four women in group 2 (one woman after 36 h, and three women after 48 h) had already started to bleed at the time the endometrial biopsy was taken; the others started bleeding after the biopsy. The bleeding lasted for 12–72 h, and all but three women reported a second bleed at the time of the expected menses. In these three women, a second episode of bleeding occurred approximately 4 wk later.

The concentration of progesterone was significantly lower at the time of biopsy in the women treated with mifepristone than in the control women (13.2 nM vs. 34.8 nM, P = 0.001). However, the levels were still significantly higher than those found during the follicular phase.

Intense PGDH immunoreactivity was observed in the cytoplasm of predominantly glandular epithelium (with a lesser degree of staining in the stromal cells) in all midluteal-phase control sections (Fig. 1A). The abundance of PGDH-positive immunostaining clearly declined, to be virtually absent by 36–48 h in both glands and in stroma (Fig. 1B). The difference in PGDH-staining scores between the control group and 36- to 48-h group were statistically significant (P < 0.05) (Fig. 2A).

In all samples (treated and untreated), COX-2 staining was localized predominantly in the endometrial glands, with no (or barely detectable) staining in the stroma (Figs. 1C and 2B). Untreated endometria in the midluteal phase showed minimal staining for COX-2 in the glandular cellular compartments. After mifepristone, a significant increase in immunoreactivity was apparent at 36–48 h (Figs. 1D and 2B) (P = 0.0018).

Steroid receptor immunostaining

As expected, there was weak staining for PR in the nuclei of stromal cells in the control samples, with minimal ER staining. AR staining was confined to stroma. There was no change in intensity or distribution of staining for steroid receptors after mifepristone (data not shown).

Discussion

Endometrial shedding and vaginal bleeding are observed after withdrawal of progesterone (for example at luteal regression) from an estrogen-primed endometrium that is subsequently exposed to progesterone (2). Similar bleeding is also seen after the pharmacological withdrawal of progesterone after administering the antiprogestrone, mifepristone, in the luteal phase of the cycle. While the endometrial morphology exhibits a marked sensitivity to mifepristone, with a 0.5-mg daily dose being the threshold dose for delay in endometrial maturation (15–17), in general, higher doses (in excess of 10 mg) are required to produce endometrial shedding and menstrual bleeding.

All women in our study reported vaginal bleeding commencing at 24–48 h after taking 200 mg mifepristone in the midluteal phase. In 13 out of the 16 subjects, this was followed by a second bleed of normal character at the expected time of the next menses. Although mifepristone significantly depressed the serum progesterone value in all women, the occurrence of a second bleed in the majority suggests only a partial luteolysis and a direct effect of mifepristone on the endometrium (18).

The current understanding advocates a central role for PCs as a trigger mechanism for menstruation (2, 3, 19, 20). The
concentration of PG in any tissue is related to its rate of synthesis and metabolism, and the variation in the endometrial release of PGs at different stages of the menstrual cycle suggests an ovarian hormonal influence. The two key enzymes that control the endometrial PG synthesis (PLA₂ and COX) seem to be under the influence of progesterone. COX enzyme exists as two isoforms produced by two different genes (COX-1 and COX-2). COX-1 is constitutively expressed, whereas COX-2 expression is modulated by a variety of stimuli and may be inhibited by progesterone in the endometrium (21). PLA₂ also seems to be present in the endometrium in two different isoforms: the calcium-dependent and inducible PLA₂, which is localized in the endometrial glands; and the calcium-independent PLA₂, which is predominantly confined to the stroma (22). Conversely, PGDH metabolizes PGs to inactive metabolites, and this enzyme is induced by progesterone (12, 13, 23). The increase in PGDH activity in a secretory endometrium that occurs in response to the rising levels of progesterone in the luteal phase (12) can be prevented by administration of antigestagens shortly after ovulation (11). Thus, progesterone is responsible for stimulating PGDH and suppressing COX-2.

In our study, treatment with mifepristone in the mid-luteal phase resulted in a decreased PGDH and an increased COX-2 expression in the endometrial glands, which was apparent at 36 h after mifepristone. This effect would be expected to be synergistic in increasing endometrial PGs (because of a synchronized suppression of metabolism, with an augmentation in the synthesis) and would lead to increased uterine activity and menstrual bleeding (2). In addition, progesterone withdrawal mediates the degradation of the endometrial extracellular matrix by inducing matrix metalloproteinases (24).

Attempts have been made to demonstrate the effects of progesterone on the endometrial PGs activity both in vitro and in vivo studies. Progesterone seems to enhance the PG biosynthetic capacity of the secretory endometrium. This is demonstrated by the in vitro studies employing cell culture techniques and by maintaining endometrial explants in culture (25, 26). Conversely, progesterone has shown to suppress the release of PGs from the endometrium (26, 27). Studies in vitro have reported a reduction of both the estradiol-stimulated and the basal PG production by progesterone (26, 28). Furthermore, during pregnancy, when progesterone levels are high, basal endometrial PG production is also reduced (29). It had been suggested that this effect might involve the inhibitory effect of progesterone on the PLA₂ activity (22).

The withdrawal of progesterone from an endometrium that has been primed with progesterone and estradiol results in an increased COX-2 expression, whereas continuing exposure to progesterone is associated with low levels of COX-2 expression (30). Evidence for increased PG activity by antagonizing progesterone also comes from in vitro data, which showed a dose-dependent induction of PGF2α release.
from endometrial stromal cells (23) and also from the in vivo observation of increased uterine contractility after mifepristone, possibly attributable to increased PGs (31–33). This is further supported by the inhibition of glandular PGDH expression seen after administration of anti progesterones in the early-luteal phase (10, 11) and during the early pregnancy (34).

A decrease in the uterine PGF2α release (33) and in the luminal expression of COX-2 had been reported after mifepristone in the early-luteal phase (35). However, in the early-luteal phase, progesterone values are relatively low, and, as a consequence, the PR and ER expression is maximal, whereas the converse is true for the midluteal phase of the cycle. Therefore, in the early-luteal phase, mifepristone may prevent the effects that are to be exerted by progesterone; whereas in the midluteal phase, it may antagonize the actions of progesterone, which, at that time, seems to be the suppression of PGs release.

Our results: are consistent with previous reports (10, 21) that demonstrated localization of PGDH and COX-2 in the glandular epithelium, and also support the in vitro evidence that glands are the main site for PG synthesis (36, 37).

There was no significant change in the level of immuno-reactivity of AR, ER, or PR after mifepristone. When given immediately after ovulation, mifepristone and onapristone prevent the progesterone-induced down-regulation of PR and ER, which normally occurs during the luteal phase (10, 38, 39). In the midluteal phase, the levels of ER and PR are already low and were unchanged at 48 h after mifepristone administration. AR staining was mainly confined to the stromal compartment and remained unchanged after mifepristone (40). In contrast, when mifepristone was given in the early-luteal phase, there was strong immunostaining for AR in the glands (41). The factors regulating the spatial and temporal expression of AR in the endometrium and its physiological role are not fully understood.

The distinct endometrial effects seen after the midluteal administration of mifepristone add to our understanding of the mechanism of menstruation. There is overwhelming evidence that PGs are involved in the process of normal menstruation (Reviewed in Ref. 3). Our results show a down-regulation of PGDH expression and a simultaneous up-regulation of COX-2 expression after administering mifepristone in the midluteal phase. Therefore, we conclude that mifepristone induces endometrial bleeding, in the midluteal phase, by a mechanism involving both PGDH and COX-2 to increase local PG levels in the endometrium.

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