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# The long-term actions of etonogestrel and levonorgestrel on decidualized and non-decidualized endometrium in a mouse model mimic some effects of progestogen-only contraceptives in women

Naomi B Morison[1], Jin Zhang[1], Tu'uhevaha J Kaitu'u-Lino[1,2], Ian S Fraser[3] and Lois A Salamonsen[1]

*[1]Prince Henry's Institute, PO Box 5152, Clayton, Victoria, Australia, [2]Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria 3168, Australia and [3]Sydney Centre for Reproductive Health Research, FPA Health, Ashfield, New South Wales 2131, Australia*

*Correspondence should be addressed to N B Morison who is now at Prince Henry's Institute, Level 4 Block E, Monash Medical Centre, 246 Clayton Rd, Clayton, Victoria 3168, Australia; Email: naomi.morison@princehenrys.org*

## Abstract

**Breakthrough bleeding (BTB), a major side effect of long-acting progestogen (p)-only contraceptives in women, is the main reason for discontinuation of their use. To understand the mechanisms of BTB, a mouse model of endometrial breakdown and repair was adapted to evaluate the effects of long-term progestogens on the endometrium. Appropriately prepared mice received either an etonogestrel (ENG)- or levonorgestrel (LNG)-releasing subdermal implant. Forty eight hours after decidualization was induced in one uterine horn the majority of tissues were highly decidualized, designated 0 day (0d). Uteri were collected subsequently at 5-day intervals (to 45d) and both decidualized and non-decidualized horns were analysed for morphological changes, leukocyte infiltration and matrix metalloproteinase expression (MMP). In decidualized horns, large blood vessels (BV) developed and disturbance of tissue integrity was observed at 5d with substantial stromal breakdown by 10d, progressing until 25d when re-epithelialization was initiated. By 45d, the tissue was restored to its pre-decidualized state but with considerable tortuosity of the luminal epithelium. Tissue remodelling was not apparent in the non-decidualized horns before 35d, when hyperproliferation of the luminal epithelium resulted in tortuosity. Changes in morphology were similar with the two progestogens, but occurred more rapidly with LNG. Apart from macrophages, few leukocytes were present in non-decidualized horns but large infiltrates of neutrophils and uterine natural killer cells (uNK) were associated with tissue breakdown in decidualized tissue, many of these cells were MMP9-positive. MMP7 was primarily associated with tissue repair. Therefore, this model mimics some of the changes observed in endometria of women using p-only contraceptives and provides an opportunity for functional studies.**

*Reproduction* (2007) **133** 309–321

## Introduction

Progestogen (p)-only contraceptives, such as the subdermal implants Implanon and Norplant or the levonorgestrel-releasing intrauterine system (LNG-IUS), provide long-term, safe and highly effective methods of birth control. However, unpredictable and often prolonged bleeding is associated with these contraceptives in approximately 30% of users and is the main reason given for their discontinuation. The mechanisms underlying this problem remain largely unknown. The different methods of delivery and local uterine concentrations of progestogen can have differing effects on the morphology of the endometrium. The LNG-IUS produces a high local concentration of progestogen and the endometrium becomes highly decidualized (Silverberg *et al.* 1986), while the slow release subdermal implants result in lower uterine progestogen concentrations and more variable endometrial morphologies. Tissue samples collected from women using Norplant have previously been characterized into three different subtypes: atrophic, shedding and decidualized (p-modified; Marsh *et al.* 1995, Vincent *et al.* 1999), although the bleeding episodes in these women did not directly correlate with the morphology of the tissue (Marsh *et al.* 1995, Vincent *et al.* 1999, Rhoton-Vlasak *et al.* 2005).

The normal menstrual cycle is guided by the steroid hormones oestrogen and progesterone which act primarily through and subsequently regulate expression


ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.01171
Online version via www.reproduction-online.org

of their specific receptors (oestrogen receptor (ER) and progesterone receptor (PR) respectively). The different methods of delivering contraceptive progestogens to the uterus result in various expression of steroid receptors in the endometrium, which may contribute to the bleeding disturbances. PR is suppressed in women using LNG-IUS, which exposes the endometrium to high levels of LNG (Critchley *et al.* 1998*a*). However, when exposed to lower uterine concentrations of progestogens, such as Implanon or Norplant, PR is high (Critchley *et al.* 1993, Macpherson *et al.* 1999). ER does not appear to be altered in Implanon users nor women using depot medroxyprogesterone acetate (MPA; Sereepapong *et al.* 2004), however, it is lower in endometrium from Norplant users when compared with normal proliferative phase endometrium (Critchley *et al.* 1993). Progestogens induce changes in ER and PR expression, which are likely to be involved in the observed histological differences and bleeding problems associated with specific p-only contraceptives.

Previous studies suggest a number of factors likely to contribute to the bleeding problems. These include an increase in number and size of small endometrial blood vessels (BV) as well as an increase in microvascular fragility (Rogers *et al.* 1993, Hickey *et al.* 1996, Pritts *et al.* 2005). Increased BV density was observed in the endometrium of women recently exposed to Norplant; however, the number of BV was not indicative of increased bleeding or spotting (Hickey *et al.* 1999). BV density was significantly increased in biopsies with an atrophic or regressed endometrial histology, however, this correlated to fewer bleeding days when compared with other histological appearances, suggesting that vessel integrity and function, not density, is important in bleeding (Hickey *et al.* 1999). Levels of vascular endothelial growth factor (VEGF) and tissue factor (TF) are also altered in the endometrium of women using Norplant (Runic *et al.* 1997, 2000, Lockwood *et al.* 2000, Schatz *et al.* 2003), and in particular TF was increased in the bleeding sites when compared with non-bleeding sites and may contribute to aberrant angiogenesis (Lockwood *et al.* 2000). Therefore, compromises in the vasculature and overall tissue integrity could play an important role in breakthrough bleeding (BTB).

Under the guidance of oestrogen and progesterone, the endometrium undergoes extensive tissue remodelling during each menstrual cycle. Synthetic steroids used in contraceptives influence the endometrium resulting in distinct morphological changes (Silverberg *et al.* 1986, Marsh *et al.* 1995, Hickey *et al.* 1999, Vincent *et al.* 1999, Rhoton-Vlasak *et al.* 2005). It is possible that molecules important for tissue remodelling will be involved in the endometrial changes observed in women using p-only contraceptives and contribute to overall tissue integrity. Many molecules thought to play important roles in tissue remodelling and angiogenesis are produced by leukocytes. During the menstrual cycle, specific subpopulations of leukocytes are recruited into the endometrium and immediately prior to menstruation can contribute up to 40% of the total cellular content of the tissue (Kamat & Isaacson 1987, Jeziorska *et al.* 1995, King 2000, Salamonsen & Lathbury 2000). Leukocytes produce a number of enzymes including matrix metalloproteinases (MMPs) which are capable of breaking down components of the extracellular matrix. MMPs are selectively upregulated just prior to menstruation (Hampton & Salamonsen 1994, Jeziorska *et al.* 1996, Zhang *et al.* 1998, Salamonsen & Woolley 1999, Zhang & Salamonsen 2002, Curry & Osteen 2003, Goffin *et al.* 2003). Furthermore, inhibition of MMP activity in explant endometrial cultures demonstrated a critical role for these enzymes in endometrial breakdown (Marbaix *et al.* 1996). Leukocytes and MMPs are also abundant in endometrium exposed to p-only contraceptives, in similar numbers to those observed in normal menstrual phase tissue and are particularly high in endometria displaying a shedding morphology (Galant *et al.* 2000, Marbaix *et al.* 2000, Vincent & Salamonsen 2000, Vincent *et al.* 2000, 2002).

The mechanisms underlying BTB and indeed menstruation and menstrual disorders are difficult to study, given that they occur only in women and a few old world primates (Shaw *et al.* 1972, Goncharov *et al.* 1976, Kaiserman-Abramof & Padykula 1989, Rasweiler 1991). The invasive nature of collecting human endometrial samples and the lack of suitable non-primate models limits investigations where these processes can be manipulated and subsequently elucidated. A major difference between women and other mammals is that very few species undergo spontaneous decidualization during a non-fertile cycle as occurs in women, therefore making direct comparisons with women difficult. We previously developed a mouse model for endometrial breakdown and repair (Brasted *et al.* 2003), in which we observed an increase in MMP expression and an influx of leukocytes (Kaitu'u *et al.* 2005) after P withdrawal, consistent with what is observed during menstruation in women. Importantly, endometrial breakdown in this model was only observed in the uterine horns in which decidualization had been induced and no changes were seen in the non-decidualized horn (Brasted *et al.* 2003, Kaitu'u *et al.* 2005). Therefore, it appears that decidualization, or at least some of the changes that accompany decidualization may be important contributors to endometrial instability.

The aims of the present study were to develop a mouse model, in which the effects of long-term administration of the progestogens used in Implanon and Norplant could be examined and manipulated. Morphology of the tissue and BV, MMP expression and leukocyte influx were examined in both the decidualised and the non-decidualized uterine horns which represent the different endometrial morphologies seen in women using p-only contraceptives.

## Materials and Methods

### *Animals*

Female C57BL/6 mice age 8–12 weeks, were obtained from Monash University Animal Services and housed under standard conditions with food and water and allowed to feed *ad libitum* with a constant light cycle of 12 h (lights on from 0800 to 2000 h). Ethics approval was granted by the Monash University/Monash Medical Centre Animal Ethics Committee B.

### *Mouse model for long-term progesterone exposure*

A previously described mouse model for endometrial breakdown and repair (Brasted *et al.* 2003, Kaitu'u *et al.* 2005) was adapted to examine the effects of long-term exposure (up to 45 days) to etonogestrel (ENG) or LNG on decidualized and non-decidualized endometrium. Briefly, ovariectomized mice received three daily (at approximately 0900 h) s.c. injections of 100 ng 17β-estradiol (Sigma Chemical Co.) in arachis oil. The mice were then rested for 3 days before insertion of one-fourth of a standard Implanon rod (containing ENG; gift from Organon, Oss, The Netherlands) or a silastic implant (0.062 inches internal diameter, Dow Corning, Midland, MI: functional size 1 cm length see (Kaitu'u *et al.* 2005)) containing LNG (Sigma, as contained in Norplant) subcutaneously into the back of the animals. Simultaneously, the mice received a single s.c. injection of 500 ng progesterone (Sigma) in arachis oil and a series of three 5 ng 17β-estradiol injections on this and the following 2 days. At approximately 1000 h on the day of the final 17β-estradiol injection, 20 µl sesame oil was injected into the lumen of uterine horn to induce artificial decidualization (stimulated horn). The left horn was left untreated (non-stimulated horn). At 48 h following the oil injection, the injected horn had undergone extensive decidualization; this was designated day 0 (0d). Mice were sacrificed at 5-day intervals from 0d until 45d (ENG) or 15d (LNG) and the uteri were collected ($n \geq 3$ at each time point). Fig. 1 details the treatment regime. All surgeries were performed under xylazine/ketamine-induced anaesthesia.

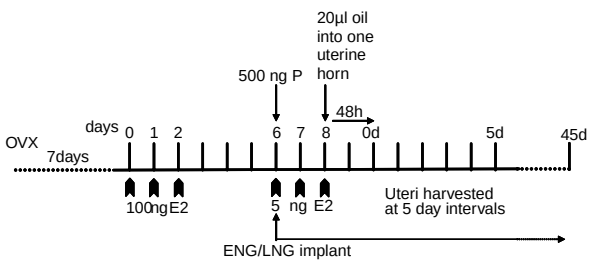


**Figure 1** A time line describing the mouse model of long-term progestogen exposure. Ovariectomized mice were allowed to recover for 7 days before receiving a series of oestrogen injections. P-implants were inserted along with a second series of oestrogen injections to prime the uterus prior for the administration of a decidualizing oil stimulus into one uterine horn. Substantial decidualization had occurred 48 h after the stimulus was given (0d) and mice were culled from this time point at 5-day intervals up to 45 days (0–45d).

### *Tissue collection*

Uteri were cleaned of fat, weighed and each horn was cut into three pieces, which were: (1) fixed overnight at 4 °C in phosphate buffered formalin, (2) fixed for 2 h at room temperature (RT) in Carnoy's fixative or (3) snap-frozen in liquid nitrogen and stored at −80 °C. Formalin- and Carnoy's-fixed tissues were subsequently processed to wax.

### *Histology and Immunohistochemistry*

Uterine cross sections (5 µm) of formalin- or Carnoy's-fixed tissues were processed through Histosol (Sigma) and a graded series of ethanol to distilled water ($dH_2O$) prior to immunostaining or histological staining. For histological analysis, hydrated sections were stained with haematoxylin and eosin using standard staining procedures and mounted using dibutyl polystyrene xylene (DPX, BDH Laboratory Supplies, Poole, England).

Immunohistochemistry was conducted to detect MMP3, MMP7 and MMP9 in cross sections of uterus at different times following decidualization as previously described (Kaitu'u *et al.* 2005). Rabbit anti-mouse MMP3 was a gift from Dr L Moons and rabbit anti-rat MMP7 was a gift from Prof. J F Woessner Jr (Yu & Woessner 2000). Goat anti-mouse MMP9 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antigen retrieval was performed on Carnoy's-fixed tissue by heating sections in 0.1 M citrate buffer for 5 min in a 700 W microwave set to medium. The slides were allowed to cool to RT and were then rinsed in $dH_2O$. Endogenous peroxidase activity was quenched by immersion of sections in 3% $H_2O_2$ in methanol for 30 min at RT. Sections were then incubated with blocking solution containing 10% normal goat serum (MMP3 and MMP7) or 20% normal horse serum (MMP9) in Tris-buffered saline (TBS) pH 7.6 for 20 min at RT. Sections were incubated for 1 h with primary antibodies diluted in 10% foetal bovine serum (FBS)/TBS to 1 µg/ml (MMP3) in the ratio of 1:300 (MMP7) or 1 µg/ml (MMP9), then washed sequentially in TBS, 0.6% (v/v) Tween 20 in TBS and thrice in TBS. Biotinylated goat anti-rabbit IgG for MMP3 and MMP7, and biotinylated horse anti-goat IgG (Dako, Glostrup, Denmark) for MMP9, diluted at a ratio of 1:200 in 10% FCS/TBS, were applied for 45 min at RT and the slides were washed as described above. For all protocols, the StreptABC horse radish peroxidase (HRP) kit and diaminobenzidine (DAB) solution (Dako) were used in accordance with the manufacturer's specifications to reveal the MMP3, -7 and -9 staining.

Immunolocalization of neutrophils was performed on formalin-fixed tissue using the rat anti-mouse monoclonal MCA771GA (Serotec, Oxford, UK) diluted to 20 μg/ml in 10% FBS/TBS. Antigen retrieval (0.1 M citrate buffer; 5 min at high level and 3 min at medium-low level in a 700 W microwave) preceded endogenous peroxidase activity quenching (3% $H_2O_2$ in methanol for 5 min). Sections were blocked with 20% normal rabbit serum for 5 min at RT then incubated with primary antibody for 30 min at RT. Sections were washed as specified for MMPs above, followed by incubation with biotinylated rabbit anti-rat IgG (Dako; 1:200 v/v in 10% FBS/TBS for 30 min). StreptABC-HRP reagent preceded colour development with DAB (Dako).

Immunolocalization of macrophages was performed on Carnoy's-fixed tissue using the rat anti-mouse F4/80 pan macrophage antibody (BMA Biomedicals, Rheinstrasse, Switzerland). Microwave antigen retrieval (10 min at high level in a 700 W microwave) was followed by an incubation with a peroxidase blocking agent (3% $H_2O_2$ in methanol for 10 min) and the primary antibody (0.81 μg/ml diluted in 10% FBS/TBS) at 37 °C for 30 min. Biotinylated rabbit anti-rat IgG (Dako; 1:200 diluted in 10% FBS/TBS) was incubated with sections for 30 min at RT, followed by StreptABC HRP reaction for 30 min and colour development using DAB (Dako) for 5 min.

To detect uterine NK cells, Carnoy's-fixed sections were incubated with peroxidase blocking agent (3% $H_2O_2$ in methanol for 5 min) prior to blocking in 20% FBS/TBS for 10 min at RT. NK cells were detected using biotinylated lectin-DBA (Sigma) diluted to 50 μg/ml in 10% FBS/TBS for 40 min at 37 °C, followed by StreptABC-HRP (Dako) for 30 min at RT and the fuchsin substrate-chromogen system (Dako) for 5 min at RT. A microcomputer imaging device (AIS: Imaging Research, Brock University, Canada) was used to assess the relative number of uNK cells (expressed as a percentage of total tissue area strongly stained) in each tissue.

Immunohistochemistry was conducted using the BV marker CD34, an affinity purified rat anti-mouse IgG (Serotec). Formalin-fixed tissues were dewaxed and rehydrated prior to quenching of endogenous peroxidase activity with 3% $H_2O_2$ in methanol for 5 min at RT. Tissues were blocked in 20% normal rabbit serum in TBS for 5 min at RT before incubating with anti-mouse CD34 (0.5 μg/ml in 10% FBS/TBS) for 30 min at 37 °C. Biotinylated rabbit anti-rat IgG (Dako; 1:200 diluted in 10% FBS/TBS) was incubated with sections for 30 min at RT, followed by StreptABC HRP reaction for 30 min and colour development using DAB (Dako) for 2 min.

Immunolocalization of ER-α was performed using an affinity purified rabbit anti-human polyclonal antibody (ER-α; H-20:sc-543, Santa Cruz Biotechnology). Carnoy's-fixed tissues were subjected to antigen retrieval (3 min at high, 5 min at medium then 1 min at high level in a 700 W microwave in 0. 1 M citrate buffer) followed by quenching of endogenous peroxidase activity (3% $H_2O_2$ in methanol for 10 min at RT) and blocking (20% normal goat serum/TBS 10 min at RT). Primary antibody was incubated with the tissues (2 μg/ml) for 60 min at 37 °C. Goat anti-rabbit/HRP conjugated polymer (Dako EnVision+System Kit) was then incubated with the tissue for 30 min at RT and visualized using DAB (Dako) for 2 min.

Immunolocalization of progesterone receptor (PR) was performed using the rabbit anti-mouse, rat and human polyclonal antibody (PR-A and PR-B; C-20:sc-593, Santa Cruz Biotechnology). Endogenous peroxidase activity was quenched in formalin-fixed sections (3% $H_2O_2$ in methanol for 10 min) followed by antigen retrieval (0.1% trypsin in 0.1% calcium chloride for 30 min at RT) and blocking in 20% normal goat serum/TBS for 10 min at RT. Sections were then incubated with primary antibody (2 μg/ml) for 1 h at 37 °C and washed with 0.6% Tween 20/TBS. Tissues were then incubated with goat anti-rabbit HRP (Polymer, Dako Envision+kit) for 30 min at RT and visualized using DAB (Dako) for 3 min.

All sections were lightly counterstained with Harris hematoxylin (Accustain; Sigma Diagnostics), dehydrated and mounted using DPX mounting medium. Negative controls were included for each tissue section by substitution of the primary antibody with a matching concentration of normal rabbit IgG for MMP3 and-7, neutrophils, macrophages, ER and PR, and normal goat IgG (Sigma) for MMP9.

### In situ *zymography*

Tissues were examined for the presence of active forms of gelatinases, by *in situ* zymography (ISZ), as previously described (Zhang & Salamonsen 2002). In brief, 7 μm frozen sections were cut on to poly-L-lysine coated slides, fixed in 10% buffered formalin for 5 min at 4 °C and washed thrice with cold TBS. The substrate (DQ™ gelatin from pig skin, fluorescein conjugate, Molecular Probes, Inc., Eugene, OR, USA) was dissolved to a final concentration of 25 μg/ml in a mixture of 2% gelatin and 2% sucrose in TBS with 0.02% sodium azide and 100 μl layered over the tissue section, covered with a coverslip and incubated in a darkened humid chamber at 37 °C for 16 h. Each section was viewed using an Olympus Corp. (Birkeroed, Denmark) fluorescent microscope with fluorescein isothiocyanate (FITC) filter.

### *Statistics*

A one way ANOVA with Tukey's *post hoc* test was used to determine differences in weights between stimulated or non-stimulated uterine horns at different time points. A $P$ value of $<0.05$ was deemed statistically significant. Results are presented as mean weight +S.E.M.

## Results

### *Changes in uterine weight*

Mice treated with ENG were culled at 5-day intervals between 0d (48 h after induction of decidualization) and 45d. The average weights (±S.E.M.) of stimulated and non-stimulated horns at each time point are shown in Fig. 2A. Weights of stimulated horns increased to a peak at 10d when they were >sixfold heavier than on 0d ($P<0.001$). The weights then decreased at 15d, but were still significantly greater than at 0d ($P<0.001$). By 20d they were no longer significantly heavier than on 0d. The non-stimulated horns showed a small but non-significant increase in weight from 0d to 25d but at 35d and 45d were significantly heavier than at 0d ($P<0.05$ and $<0.01$ respectively).

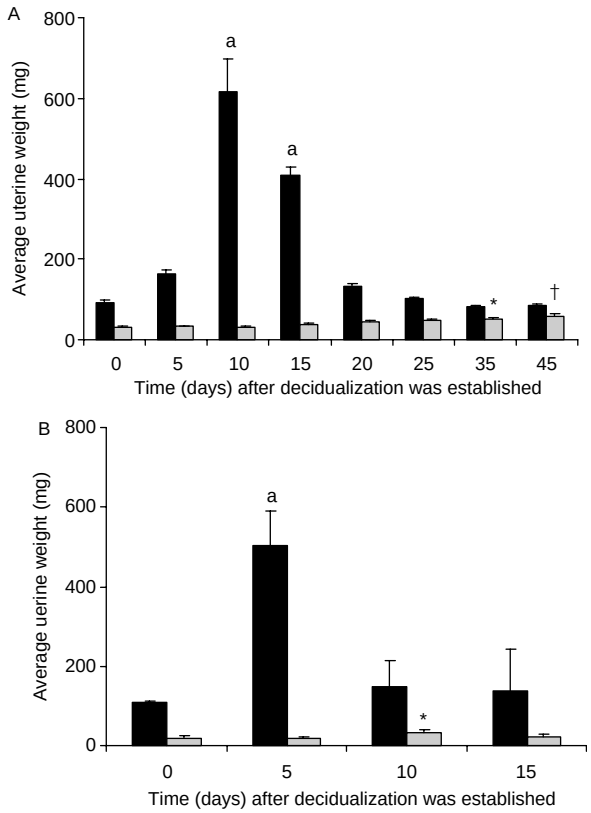


**Figure 2** Average uterine weights of stimulated and non-stimulated horns. Uteri were collected from mice treated with etonogestrel (A) or levonorgestrel (B) at 0–45d and dissected into stimulated (dark bars) and non-stimulated (light bars) horns. These horns were weighed separately and average wet weight is shown in milligrams (+S.E.M.). (A) Etonogestrel; the stimulated horn was significantly increased in weight at 10d and 15d when compared with 0d (a=$P<0.001$) before decreasing. The non-stimulated horn remained a consistent weight until 35 and 45d when significant increase from 0d was observed (*$P<0.05$ and †$P<0.01$ respectively). (B) Levonorgestrel; the stimulated horn significantly increased in weight from 0 to 5d (a=$P<0.001$) before decreasing. The weight of the non-stimulated horn at 10d was significantly different to the non-stimulated horn at 0d (*=$P<0.05$).

The stimulated horns collected from mice treated with LNG displayed a similar significant increase in weight to those collected from ENG-treated mice (~fivefold increase from 0d to 5d, $P<0.001$, Fig. 2B). However, the peak weight was observed at 5d (when compared with 10d for ENG mice) and at 10d the weight of the stimulated horn had decreased and was not significantly different from 0d. Again there was very little variation in the weights of the non-stimulated horn; only at 10d there was a significant increase from 0d ($P<0.05$).

### *Morphological changes*

The morphology of the uterine tissue was assessed at each time point by haematoxylin and eosin staining. Representative photomicrographs are shown in Fig. 3. At 0d in the stimulated horn, stromal expansion and differentiation of the decidual cells was apparent. Closure of the lumen was observed and small glands were seen in the basal zone (Fig. 3, 0d morphology). At 5d, large BV were obvious in the central decidual zone and confirmed by CD34 staining of endothelial cells (Fig. 3). These were not detected at 0d demonstrating a paucity of such vessels in early decidua. At 5d, there was also some loss of structural integrity and red blood cells (RBC), and leukocytes were evident in the decidual zone close to BV (Fig. 3, 5d). At 10d, there was clear evidence of stromal breakdown, with large areas of destruction within the decidual zone and an abundance of RBC in the lumen (Fig. 3, 10d). Structural integrity of the BV was compromised at 15d (Fig. 3). This breakdown progressed from 15d–25d (Fig. 3) and in some areas the decidual zone appeared to part almost completely from the myometrium (Fig. 3, 20d). By 25d, there was some evidence of re-epithelialization and large numbers of RBC along with cellular debris were isolated within the lumen (Fig. 3). At 35d, the debris was almost entirely cleared from the lumen and re-epithelialization was often observed to be complete (Fig. 3, 35d). At 45d, the stimulated horn had completely restored to a pre-decidualized state (Fig. 3, 45d), however, the luminal epithelium appeared highly tortuous. At these later time points after tissue restoration (25–25d), BV staining was observed throughout the stroma, similar to that of the non-stimulated horns at these times (data not shown).

In comparison, the non-stimulated horn (Fig. 4) showed no signs of stromal differentiation at 0d with glands scattered throughout the stroma (Fig. 4, 0d). Supporting the lack of change observed in the weights of the non-stimulated horns, these tissues remained largely unchanged in morphology from 0d to 25d (Fig. 4). There appeared to be some stromal oedema at 25d which then increased overtime. Furthermore, at 35d and 45d (Fig. 4) the luminal epithelium of the non-stimulated horn became more tortuous, resembling that of the stimulated horn at these time points and possibly contributing to the slight change in weights observed at the later time points.

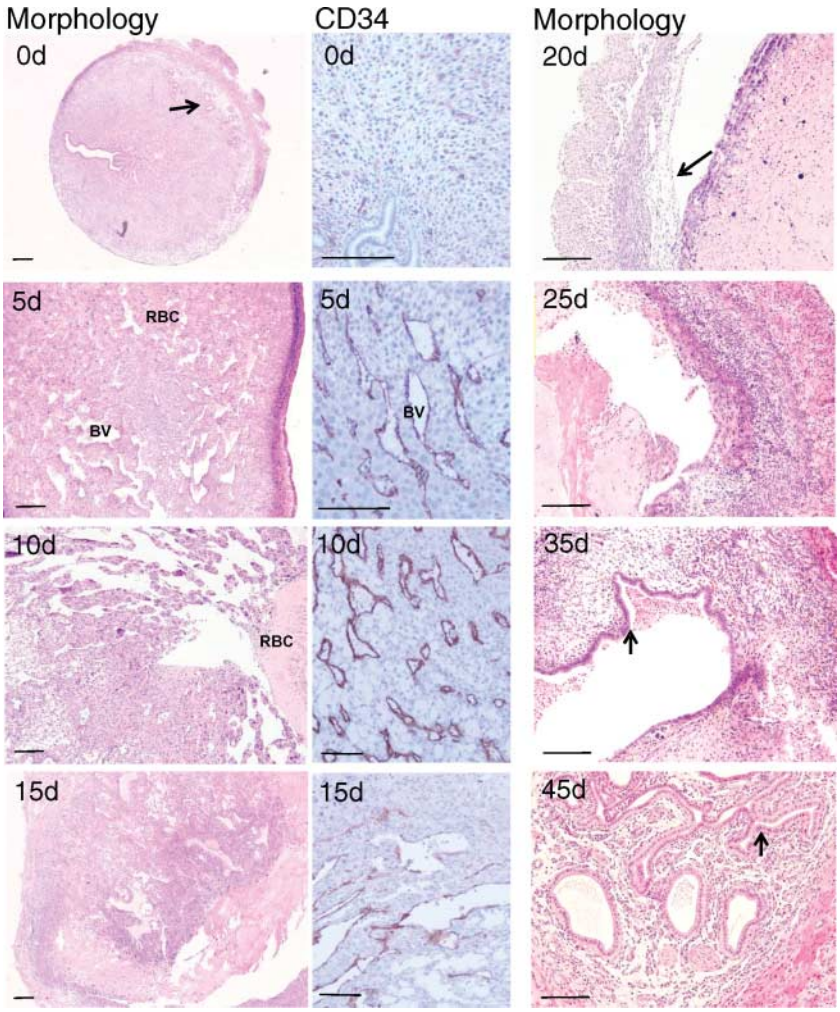


**Figure 3** Morphology and the development of blood vessels in the stimulated horn from etonogestrel-treated mice. Cross sections of stimulated uterine horns collected at 0–45 days (d) from mice treated with etonogestrel were stained with haematoxylin and eosin. Blood vessels (BV) were identified in some sections by immunohistochemistry for CD34. The stimulated horn was highly decidualized at d0 with glands located in the basal area (arrow). By 5d, large BV had appeared (as shown by CD34 staining of endothelial cells) along with areas containing red blood cells (RBC). The process of breakdown began around 10d. BV began to lose integrity at 15d (CD34, 15d) and breakdown continued until 25d. At 20d the decidual zone had begun to break away from the myometrium (arrow) and repair of the epithelium was observed around 25d. By 35d the repair was almost complete (arrow) and by 45d the repaired epithelium was very tortuous. Scale bars; CD34 stained sections = 50 μm, morphology = 100 μm.

The morphology of the uteri from LNG-treated animals did not differ from that in the ENG-treated animals. In the stimulated horn (Fig. 5A), there was closure of the lumen at 0d with signs of stromal decidualization and glands were located in the basal area. At 5 and 10d, large BV, similar to those stained for endothelial cells in Fig. 3, were apparent and there was loss of structural integrity and evidence of tissue breakdown. At 15d there were some signs of re-epithelialization and tissue regeneration, reflecting the more rapid change in uterine weight in these animals when compared with those treated with ENG. In contrast, the non-stimulated horn (Fig. 5B) showed no signs of decidualization, but importantly at 15d there was increased tortuosity of the luminal epithelium and stromal oedema similar to that seen at the later time points in ENG-treated mice.

### *Oestrogen and progesterone receptors*

In the stimulated horns collected from ENG-treated mice, nuclear ER staining was present in cells throughout the stroma in tissues that were not yet breaking down (0–10d, representative photomicrograph Fig. 6A, 5d). By 15d, when tissue integrity was clearly compromised, immunoreactive ER was lost from stromal cells and localized to the nuclei of large BV and glands (not shown). At later time points when the tissue was undergoing repair, ER was detected in the endometrial glands located in the basal area of the tissue (Fig. 6A, 35d).

Very little PR staining was detected in any cells at any of the time points (not shown). However, positive control uterine tissue collected from normal cycling mice showed a high level of staining throughout the endometrium (data not shown), confirming that PR are downregulated in decidualized endometrium exposed to long-term-ENG.

### *Matrix metalloproteinases*

#### *MMP9*

At 0d, isolated MMP9-positive cells were scattered throughout the decidual zone of the stimulated horns collected from ENG treated mice (Fig. 6B). Numbers

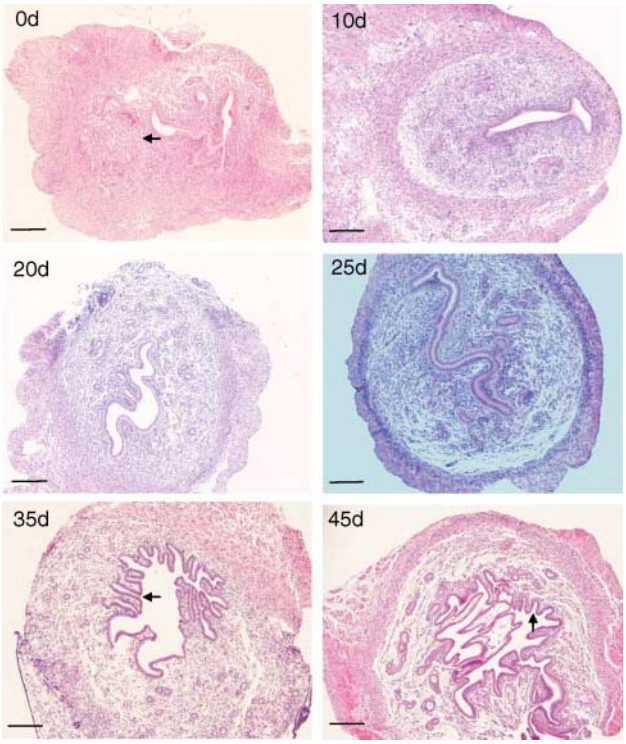


**Figure 4** Morphology of the non-stimulated horn from etonogestrel-treated mice. Cross sections of non-stimulated uterine horns collected from mice treated with etonogestrel were stained with haematoxylin and eosin. The endometrium showed no signs of decidualization at 0 days (d) with glands observed throughout the stroma (0d, arrow). The morphology of the non-stimulated horn remained largely unchanged from 0–25d although some evidence of stromal oedema began at 20d. At 35 and 45d, the luminal epithelium was very tortuous (arrows) similar to the epithelium after repair in the stimulated horns (Fig. 3, 45d). Scale bars = 200 μm.

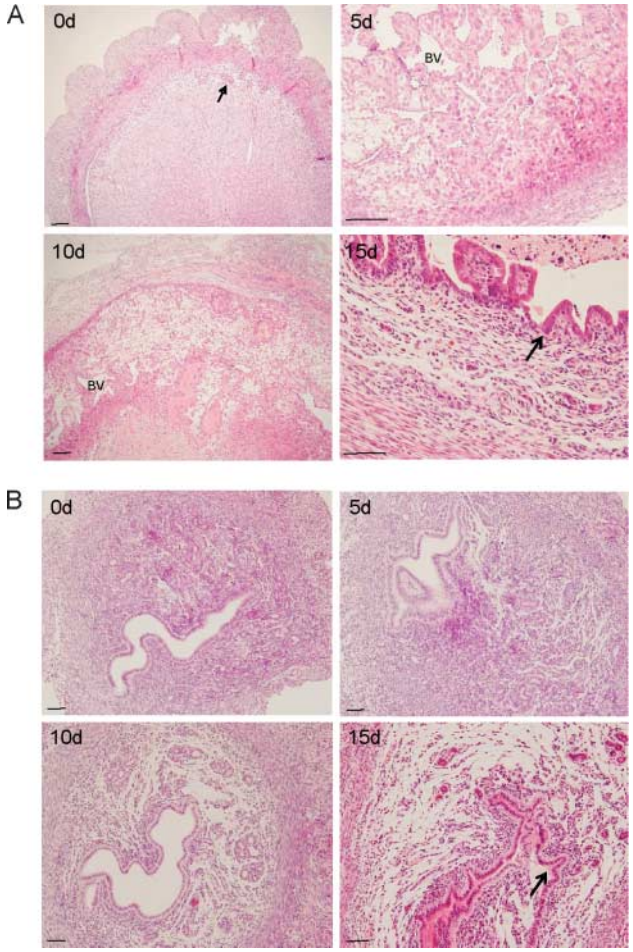


**Figure 5** Morphology of both stimulated and non-stimulated horns from levonorgestrel-treated mice. Cross sections of stimulated (A) and non-stimulated (B) uterine horns collected from mice treated with levonorgestrel were stained with haematoxylin and eosin. The stimulated horn was highly decidualized at 0 days (d) with glands observed in the basal area (arrow). Large thin-walled blood vessels (BV), similar in morphology to those seen in ENG-treated animals, were observed at 5 and 10d with evidence of epithelial repair at 15d (arrow). The non-stimulated horn (B) altered very little throughout the time course with no evidence of stromal differentiation or tissue breakdown. Similar to ENG-treated animals, at 15d the luminal epithelium appeared very tortuous (arrow) and stromal oedmea was apparent at 10 and 15d. Scale bars = 50 μm.

were greatly increased at 5d, particularly in areas of breakdown and maintained until 20d (representative photomicrograph Fig. 6B). At 35d, the number of MMP9-positive cells dramatically decreased and only a few isolated positive cells remained (not shown). Gelatinase activity (which includes MMP9 and -2 activities; green fluorescence) was confirmed using ISZ in these tissues at 5d (Fig. 6B 5d, ISZ). No MMP9-positive cells were detected in the non-stimulated control horns at any time point (not shown).

*MMP7*

Isolated MMP7-positive cells were observed in the stroma and the breakdown areas in the stimulated horns from 0–20d (representative photomicrograph Fig. 6C). At the later time points (25–35d), MMP7 was mainly associated with the process of repair and re-epithelialization and was often observed near areas of newly formed epithelium (Fig. 6C, 35d). At 45d, when the tissue had completely regenerated, there was no MMP7-positive staining (not shown). MMP7 staining was not consistently detected in the non-stimulated horn at any time point, although there were occasional isolated positive cells in some sections (not shown).

*MMP3*

Immunopositive staining for MMP3 was present throughout the time course in the stimulated uterine horns. Diffuse interstitial staining was observed at isolated foci within the decidua and did not appear to be altered during tissue breakdown or repair (representative photomicrograph Fig. 6D). There was no significant MMP3 staining in the non-stimulted horn at any time point (not shown).

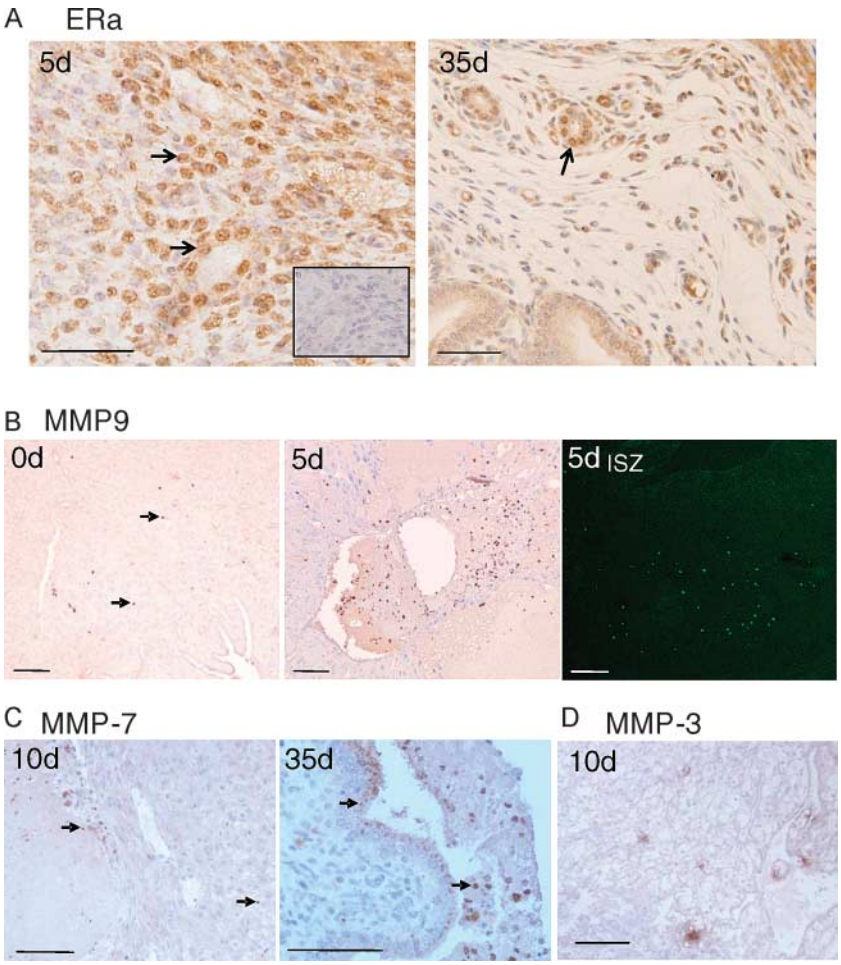


**Figure 6** Oestrogen receptor staining in the stimulated horn of etonogestrel-treated mice. Cross sections of uterine horns collected from mice treated with etonogestrel were immunostained for oestrogen receptor α (ER). ER-positive cells were observed throughout the stroma in decidual tissue (A 5d, arrows indicating nuclear staining) and during the early stages of tissue breakdown. There was a reduction in staining as breakdown progressed (not shown); however, during tissue repair, staining was detected in glands (A 35d, arrow) in the basal area. Inset = negative control. Scale bars = 50 μm. MMP localization and gelatinase activity in the stimulated horn of etonogestrel-treated mice. Cross sections of the stimulated horns from mice treated with etonogestrel were analysed by immunohistochemistry for the presence of MMP9 (B), MMP7 (C) and MMP3 (D) as well as *in situ* zymography to detect gelatinase activity (B, ISZ). Immunopositive MMP9 (B) was detected in a small number of cells in decidualized tissue (0d arrow). Numbers of positive cells increased during tissue breakdown (5d) and were maintained up to 20d (not shown). Immunopositive MMP7 (C) was detected in isolated cells in decidual and breaking down tissue (10d, arrows). Numbers were increased at sites of re-epithelialization during tissue repair (35d, arrow). Immunopositive MMP3 (D) was detected in isolated instances throughout the time course (representative 10d). Gelatinase activity was readily detectable at 5d, coincident with high numbers of immunopositive MMP9 (B, ISZ). Scale bars = 100 μm.

### *Leukocytes*

In the tissue collected from ENG-treated mice, very few uNK cells were observed in the non-stimulated horn or in decidualized endometrium at 0d (not shown). Immunostaining revealed a dramatic increase in uNK cells in the stimulated horn at 5d, particularly in the mesometrial region containing large vessels (Fig. 7A). The uNK cells were located throughout the stroma in this area, with no obvious relationship to BV (Fig. 7A). By 10d, the number of uNK cells had not greatly increased further but they were now mainly located within the breakdown area (Fig. 7A, 10d). At 15d, uNK cell numbers decreased, coincident with the onset of tissue repair (not shown). Semi-quantitative analysis of the proportion of the tissue stained for uNK cells showed that approximately 20% of the tissue stained for uNK cells at 5 and 10d (Fig. 8). This decreased to approximately 5% by 15d. The level of staining at other time points was below the level of detection.

Very few neutrophils were detected in the non-stimulated horns at any time points. Neutrophils were present in low numbers in the stimulated horn at 0d, and increased only slightly throughout the tissue at 5d (Fig. 7B). These cells were mostly located within BV with a few isolated cells located within the stroma. At 10d, when tissue breakdown was first apparent, large numbers of neutrophils were located within these breakdown areas and isolated neutrophils were detected in the surrounding decidua (Fig. 7B). High numbers of neutrophils persisted at 15d, but these were located almost entirely within the cellular debris (not shown). After 15d, coincident with the onset of tissue repair, the numbers of neutrophils decreased until very few remained at 45d when the debris had been entirely cleared from the lumen and the tissue had completely repaired (not shown).

Macrophages were present in the stimulated horn at 0d in the basal area and near the lumen, and were consistent in number from 0–10d (representative photomicrograph Fig. 7C). At later time points, macrophage numbers increased at sites of tissue repair (Fig. 7C, 20 and 35d).

In the non-stimulated horn, macrophages were abundant throughout the time course. These were located within the stroma and often underlying the luminal epithelium (Fig. 7D).

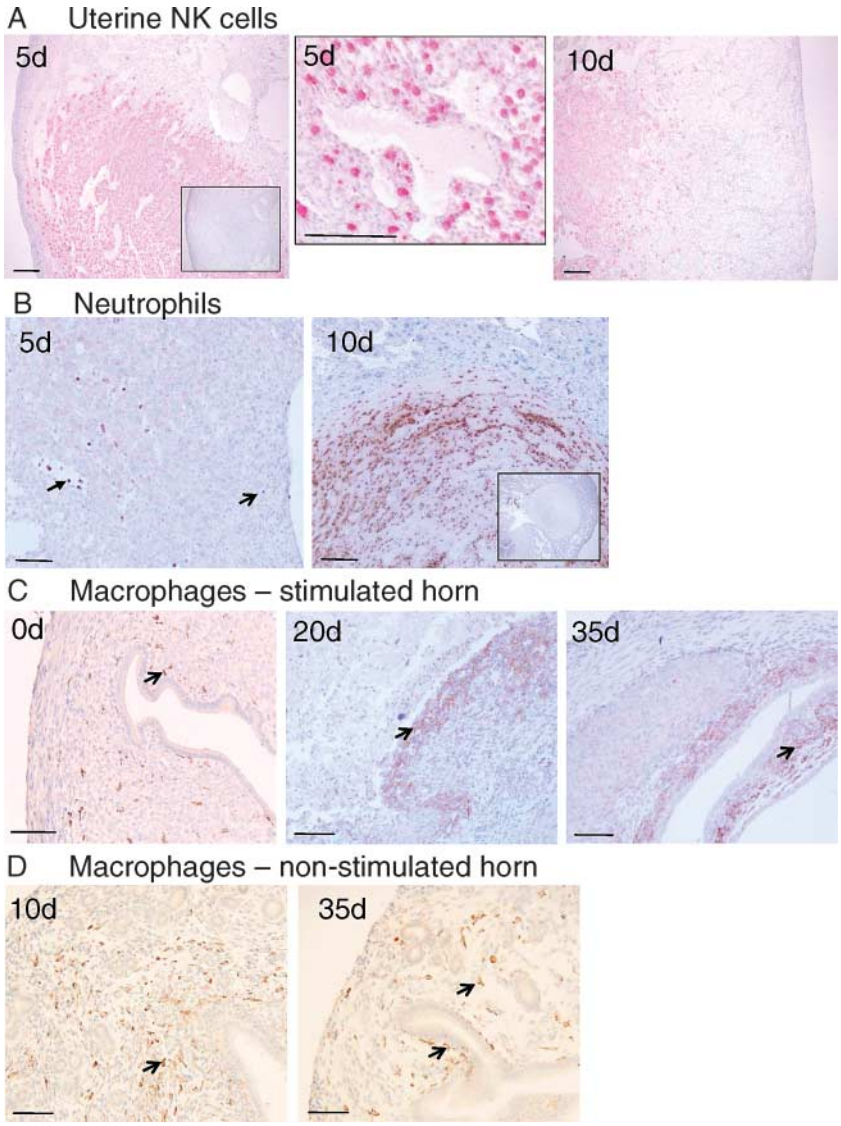


**Figure 7** Leukocyte localization in stimulated and non-stimulated horns from etonogestrel-treated mice. Stimulated (A–C) and non-stimulated (D) uterine horns were collected from mice treated with etonogestrel at various time points. Cross sections were analysed by immunohistochemistry for uterine natural killer (uNK) cells (A), neutrophils (B) and macrophages (C and D). uNK cells (A) were observed in high numbers in areas of tissue breakdown after 5 and 10d (inset; negative control). The higher power image shows their relationship to BV. Isolated neutrophils (B) were seen throughout the tissue at 5d (arrows), and increased in number in the tissue undergoing breakdown at 10 and 15d (inset; negative control). In the stimulated horn (C), macrophages were observed throughout the decidualized (0d, arrow) and subsequently breaking down tissue and were associated with re-epithelialization during tissue repair (20 and 35d arrows). In the non-stimulated horn, isolated macrophages were observed throughout the time course (D, arrows). Scale bars = 50 μm.

## Discussion

This study has adapted a previously described mouse model of endometrial breakdown and repair (Brasted *et al.* 2003) to investigate the long-term effects of progestogens contained in certain p-only contraceptives on the endometrium. A range of endometrial morphologies are found in women using long-term progestogens, including atrophic, highly decidualized and tissue undergoing breakdown (Silverberg *et al.* 1986, Critchley *et al.* 1998*a*, Vincent *et al.* 1999, Phillips *et al.* 2003). For this reason, we subjected one uterine horn of mice treated with either ENG or LNG to a decidualizing stimulus, while the other remained non-decidualized. The study demonstrated similarities between this mouse model and what is observed in tissue collected from women using p-only contraceptives. The horn in which decidualization was induced, underwent breakdown and repair, while the

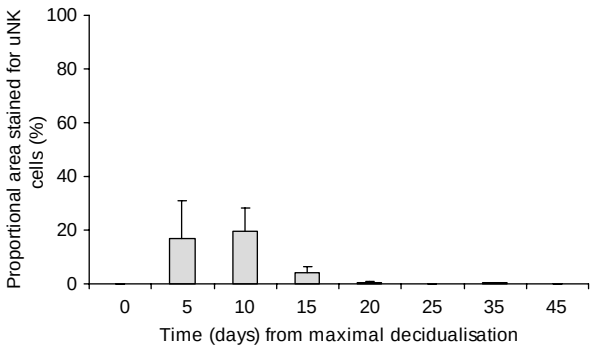


**Figure 8** Proportional area of tissue stained with uterine natural killer cells: stimulated horn of etonogestrel-treated mice. Cross sections of the stimulated horns from mice treated with etonogestrel were analysed using the analytical imaging system (AIS) to determine the proportional area of the stimulated horn stained by uNK cells. Proportional area (percentage + S.E.M.) was determined at each time point ($n > 3$ sections for each time point).

non-decidualized horn slowly increased in weight but did not undergo major remodelling. In both horns, the luminal epithelium became highly tortuous with prolonged progestogen exposure. Prior to the breakdown of the decidualized endometrium, there was an increase in the number and size of BV and a transient influx of neutrophils and uNK cells that persisted throughout the breakdown period. Immunopositive MMP9 cells were observed in high numbers in areas of breakdown, while MMP7 and -3 were consistently observed in decidualized, breaking down and repairing tissue. MMP3 was present in foci throughout the tissue at all time points. Although MMP7 was initially localized to isolated cells within the stroma and breakdown area, in repairing tissue it was mainly localized to regenerating epithelium.

In women using p-only contraceptives, endometrial morphology varies with the type of contraceptive used and the method of progestogen delivery. When high local concentrations of progestogen are delivered to the endometrium using an intrauterine device, such as the LNG-IUS, extensive decidualization and epithelial atrophy were observed and the tissue remains decidualized until the device is removed up to several years later (Silverberg *et al.* 1986, Critchley *et al.* 1998*a*, 1998*b*, Phillips *et al.* 2003). However, the decidua induced by artificial stimulation (such as oil injection) in mice, has a finite life span of approximately 8 days before spontaneous breakdown occurs. This time can be increased to 14 days following the administration of progesterone (Hirabayashi *et al.* 1999), as seen in mice treated with ENG in the present study. However, in mice treated with LNG, the weights of the stimulated horns were reduced at 10d, highlighting the differences between different progestogens. Furthermore, the finite life-span of the artificial decidua in mice is a clear and fundamental difference between the endometrium of mice and women.

Subdermal implants, such as Norplant or Implanon, result in lower intrauterine progestogen concentrations and produce a more variable effect on endometrial morphology (Marsh *et al.* 1995, Vincent *et al.* 1999). Tissue from Norplant users has been morphologically subclassified into atrophic (narrow proliferative-type epithelial glands), shedding and progestogen-modified (highly decidualized) endometrium (Marsh *et al.* 1995, Vincent *et al.* 1999). Thus, the non-decidualized horn of the mouse model would be expected to more closely represent the non-decidualized morphologies. However, neither atrophy nor shedding was observed and the most striking feature of non-decidualized horns was the hyperproliferation of the luminal epithelium, resulting in tortuosity, after 25d. This was also observed in the restored but originally-stimulated horn.

Two different progestogens were compared in this study. Although the overall morphology of the tissue was very similar in both LNG- and ENG-treated animals, the processes appeared to be retarded in ENG-treated mice highlighting the different effects of specific progestogens. While endometrial morphology has been well described in Norplant exposed tissue, such information for Implanon-exposed tissue is very limited and the different effects of the two progestogens on endometrial morphology require further investigation.

BTB is thought to arise from superficial vessels within the endometrium which increase in number and fragility in Norplant users (Hickey *et al.* 1996, Hickey & Fraser 2002). We also observed substantial changes in BV in the mouse model. Large BV with thin walls appeared after 5 days and continued to increase in number and luminal volume over the subsequent 15 days. However, at 15 days these vessels appeared to lose structural integrity and become leaky. Areas of condensed RBC within the stroma, indicative of haemorrhage, were evident. Studies using a different mouse model have also reported changes in endometrial vasculature in response to either MPA or LNG (Girling *et al.* 2004). However, in this model, normal cycling mice were exposed to progestogens in the absence of any decidualizing stimulus. Despite the differences between the models, the changes observed in the endometrial vasculature highlight the important role that progestogens exert on vessel size and fragility, even in the absence of decidualization.

The normal menstrual cycle is driven by the steroid hormones, oestrogen and progesterone, which function via specific receptors. PR is expressed in the stroma throughout the menstrual cycle with a slight decrease during the menstrual phase (Critchley *et al.* 1993). In the glands, PR protein is maximal in the mid-late proliferative and early secretory phases, then downregulated during the late secretory phase, remaining low during the menstrual and early proliferative phase (Critchley *et al.* 1993, Ingamells *et al.* 1996). In Norplant users, immunoreactive PR is higher in the stroma when compared with normal endometrial samples at any stage of the cycle and is also high in glands (Critchley *et al.* 1993). ER however, is expressed at lower levels in the stroma and glands in Norplant users when compared with normal proliferative phase endometrium (Critchley *et al.* 1993). Neither PR nor ER is altered in women using depot MPA experiencing frequent bleeding episodes when compared with women who experienced amenorrhea (Sereepapong *et al.* 2004). However, in women using LNG-IUS, PR is suppressed (Critchley *et al.* 1998*a*). This is likely a result of the high endometrial concentration of progestogen and corresponds to our observations in this mouse model in which the dose of progestogen was high. Therefore, our studies support those of others, showing that ER and PR expression can be altered by progestogens. It is also important to note that the mice in the present study were ovariectomized although the diet contained phytoestrogens which have been shown to affect the uterus (Britt *et al.* 2005).

Uterine NK cells appear in human endometrium in association with the induction and continuation of

decidualization (Marshall & Jones 1988, Croy & Kassouf 1989, King *et al.* 1989) and have also been observed in high numbers in highly decidualized progestogen-modified human endometrium (Critchley *et al.* 1998*b*). Furthermore, high numbers of uNK cells have been associated with the bleeding episodes in menopausal women receiving hormone replacement therapy (Hickey *et al.* 2005). This is consistent with the high numbers of uNK cells in the highly decidualized tissue prior to breakdown in this mouse model. In mice, uNK cells are a major source of VEGF (Wang *et al.* 2003) and studies in uNK cell-deficient mice have defined a role for these cells in the modification of spiral arterioles during pregnancy (Guimond *et al.* 1997, Croy *et al.* 2003). In the present study, we showed that uNK cells were present in high numbers in tissue that contained large thin-walled BV. Although a functional relationship remains to be established, it is likely that these cells are an important contributor to the BV remodelling in this model.

Consistent with observations in normal pre-menstrual tissue, we observed high numbers of neutrophils infiltrating the breakdown areas of the mouse tissue. These cells also produce many cytokines and proteases including elastase and MMPs, such as MMP9 and MMP7, that are thought to contribute to tissue breakdown (Kamat & Isaacson 1987, Jeziorska *et al.* 1995, King 2000, Salamonsen & Lathbury 2000, Zhang & Salamonsen 2002). The presence of these cells in the breakdown areas in this model supports this role and is in agreement with observations from progestogen-exposed human tissue where elevated numbers of neutrophils are also seen in areas of breakdown (Vincent *et al.* 1999).

MMPs degrade components of the extracellular matrix as well as regulating their own activity and that of other bioactive molecules. Inhibition of MMP activity in human explant endometrial cultures demonstrated a critical role for these enzymes in endometrial breakdown (Marbaix *et al.* 1996). MMPs are also postulated to have an important role in irregular bleeding with p-only contraceptive use (Skinner *et al.* 1999, Vincent *et al.* 1999, 2000, Vincent & Salamonsen 2000). In the present model, immunopositive MMP9 was observed in low abundance in decidualized tissue with an increase observed in association with breakdown. This expression pattern supports previous data that showed an upregulation of MMP9 in endometrial tissue, with a shedding morphology collected from Norplant users and in women who experience dysfunctional uterine bleeding, consistent with a likely role in tissue breakdown (Vincent *et al.* 1999, Galant *et al.* 2004). MMP3 was consistently expressed at foci during the process of tissue breakdown and repair in the decidualized horn, supporting human studies showing that MMP3 is elevated in endometrium from women using Norplant, DepoProvera and LNG-IUS (Vincent *et al.* 2000, Galant *et al.* 2004, Oliveira-Ribeiro *et al.* 2004). Interestingly, MMP7 was mainly associated with the process of repair and re-epithelialization and was predominantly localized to the regenerating epithelium. This staining pattern supports previous studies showing MMP7 expression is highly important for wound repair and epithelial cell migration in the epithelial tissue of damaged airways (Parks *et al.* 2001). MMP7 has also been shown to be upregulated in intestinal re-epithelialization (Salmela *et al.* 2004). The results from the present study would support such a role for epithelial-associated MMP7 as it was localized to newly formed epithelium in the repairing endometrium and is consistent with a similar location in our mouse model for endometrial breakdown and repair, a model that mimics some key features of menstruation in women (Kaitu'u *et al.* 2005). Although it was shown in the breakdown and repair mouse model that MMPs were not the key contributors to these processes, investigations into the functional role of MMPs in the long-term p-only model have been unsuccessful as we were unable to completely block MMP activity using specific inhibitors. Therefore, we can only speculate on their role in this model based on localization, activity and previous studies. The localization pattern of MMP expression is consistent with the present hypothesis that they play a role in endometrial breakdown and repair.

While this model mimics some characteristics of endometrium of women using p-only contraceptives, we acknowledge some limitations. Very high doses of progestogen were administered in the present model and to reduce this may prove difficult, particularly for ENG which was provided as Implanon rods, prepared for women, not mice and which when cut, present complex pharmacodynamics. In addition, there appear to be fundamental differences between decidua in mice and in women, both with respect to their different finite lives and to their mechanisms of induction.

In summary, the present study describes a mouse model for the study of the effects of long-term progestogen exposure on the endometrium and demonstrates some similar characteristics to those observed in the endometrium of women using p-only contraceptives. Studies in women are limited for obvious reasons and this model hence offers an opportunity for elucidating the potential functions of key modulators of endometrial fragility in BTB.

## Acknowledgements

We thank Dr Devora Lieberman of Organon for the provision of the Implanon rods. This project received financial support from the National Institute of Health (Grant no. HD43192) and the UND/UNFPA/WHO/World Band Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization. LAS was funded by the NH&MRC of Australia (#388901). TJK-L was a recipient of an Australian Postgraduate Association scholarship. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 8 March 2006
First decision 31 March 2006
Revised manuscript received 16 June 2006
Accepted 29 September 2006