A critical period of progesterone withdrawal precedes endometrial breakdown and shedding in mouse menstrual-like model

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STUDY QUESTION: Is there a critical period of progesterone (P4) withdrawal in a mouse menstrual-like model, and at what time after P4 withdrawal endometrial breakdown become irreversible?

STUDY ANSWER: Our results showed that a 12–16 h critical period of P4 withdrawal exists in the mouse menstrual-like model.

WHAT IS KNOWN ALREADY: P4 withdrawal is the trigger for endometrial breakdown and shedding during menstruation. To date, the molecular mechanisms responsible for endometrial breakdown have not been fully elucidated. In an ovariectomized macaque model, P4 replacement could reduce or block menses during a period of 36–48 h after P4 withdrawal, but after this, P4 supplementation did not reduce or block menses. Thus, in the macaque, a critical period of P4 withdrawal lasting 36–48 h exists before menses.

STUDY DESIGN, SIZE, DURATION: We created a mouse menstrual-like model and restored P4 at four time points. The total number of mice was 120 and the duration of treatment was 26 days.

PARTICIPANTS, SETTING, METHODS: A mouse menstrual model was characterized by endometrial morphology and plasma P4 levels. P4 was then replaced at 8, 12, 16 and 20 h after the removal of P4 implants. Vaginal smears, endometrial morphology, plasma P4 levels and expression patterns of matrix metalloproteinases (MMP-2, MMP-3, MMP-9, MMP-10, MMP-11 and MMP-13) were investigated.

MAIN RESULTS AND THE ROLE OF CHANGE: Replacement of P4 at 8 and 12 h blocked menstrual-like bleeding and endometrial shedding; however, replacement at 16 and 20 h did not suppress bleeding or shedding. Furthermore, P4 replacement at 12 h inhibited the expression of all latent or active MMPs; however, replacement at 16 h inhibited only pMMP-13.

LIMITATIONS, REASONS FOR CAUTION: Although determination of the critical period in vivo using a mouse model was successfully demonstrated, the mechanisms of P4 regulation need to be further explored.

WIDER IMPLICATIONS OF THE FINDINGS: The experimental opportunities provided by the mouse model will facilitate understanding the role of P4 in the regulation of menstruation and help to identify new targets for the clinical intervention of menstrual disorders.

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**Introduction**

The physiological withdrawal of progesterone (P4) from an estrogen–P4-primed endometrium (which occurs with the demise of the corpus luteum) is the triggering event for endometrial breakdown and shedding. P4 withdrawal is the triggering factor for a cascade of molecular and cellular interactions, and matrix metalloproteinases (MMPs) are the final effectors of menstruation.

In Kelly’s hypothesis on menstruation, two stages after P4 withdrawal were proposed: a P4-dependent and a P4-independent stage (Kelly et al., 2001). In the P4-dependent stage, P4 withdrawal causes initiation of vasoconstriction and local cytokine production, and these are mediated through P4 receptor (PR) positive perivascular cells. In the subsequent P4-independent stage, consequences of earlier P4 withdrawal activate downstream lytic events, which are mediated by PR-negative leukocytes and possibly other cells. A ‘critical period’ exists between these two stages and after this critical period has passed, replacing P4 could not block tissue breakdown, sloughing and bleeding. This critical period in endometrial breakdown and shedding is worth investigating to understand the mechanism of endometrial shedding during menstruation in the presence or absence of regulation by P4.

In the ovariectomized macaque menstrual model, menstruation can be induced by withdrawing P4. Restoring P4 could prevent frank menses if done up to 36–40 h after P4 withdrawal and could attenuate the menses for up to 48 h after P4 withdrawal. Thus, in the macaque model, the critical period of P4 withdrawal before menses lasts 36–48 h (Slayden and Brenner, 2006). This critical period divides P4 withdrawal into the P4-dependent and P4-independent phases of endometrial breakdown, and also provides the proper timing for potential interventions for relevant pathological complications.

The unavailability of non-human primate models has constrained the study of endometrial breakdown. Fortunately, the mouse menstrual-like model provides a convenient tool to investigate the mechanisms underlying the endometrial breakdown process (Finn and Pope, 1984; Brasted et al., 2003; Xu et al., 2007; Rudolph et al., 2012). As a mammalian animal model, mice have well-understood genetics and are suitable for advanced ‘omics’ techniques.

Finn and Pope (1984) established a model for menstruation in mouse. Following a sequential administration of estrogen and P4, endometrial decidualization was induced by the injection of oil into the uterine lumen of ovariectomized mice. Subsequently, the prevention of P4 supply led to endometrial bleeding that was similar to human menstruation. Brasted et al. (2003) optimized the mouse model, achieving more reproducible results. The mouse menstrual-like models developed using different methods have been widely accepted in the study of endometrial breakdown and also have been validated for their reliability (Finn and Pope, 1984; Brasted et al., 2003; Kaitu’U et al., 2005; Kaitu’U-Lino et al., 2007; Xu et al., 2007; Fan et al., 2008; Rudolph et al., 2012). However, the critical period of P4 withdrawal in the mouse menstrual-like model has not been determined to date.

In this study, we hypothesized that a critical period of P4 withdrawal exists before endometrial breakdown in the mouse menstrual-like model. We characterized a P4 replacement strategy in the mouse model by physiological P4 withdrawal and determined the critical period of P4 withdrawal in the endometrium by investigating vaginal bleedings, morphological changes in the uterine endometrium, P4 levels and the expression of matrix metalloproteinases (MMPs, MMP-2, MMP-3, MMP-9, MMP-10, MMP-11 and MMP-13).

**Materials and Methods**

**Animals**

Female virgin C57 BL/6j mice (8–12 weeks old) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. Mice were maintained under controlled light (on from 06:00 to 18:00 h) and temperature (21 ± 1°C) conditions and allowed free access to diet and water. All experimental procedures were approved by the Animal Ethics Committee of the National Research Institute for Family Planning.

**Experimental designs for the induction of the mouse menstrual-like model and P4 replacement**

The procedures for the establishment of the mouse menstrual-like model are outlined in Fig. 1A and described by Brasted et al. (2003). Briefly, mice were ovariectomized under anaesthesia and allowed to recover in 2 weeks. All mice were subcutaneously (s.c.) injected daily with 100 ng of 17β-estradiol (E2) (Alfa Aesar Inc., Heysham, UK) in arachis oil at 09:30 h on Days 1, 2 and 3 of the study. On Day 7, P4 implants were inserted s.c. into the back of each mouse at 09:30 h and, at the same time, 50 μg of P4 (Sigma-Aldrich Inc., St. Louis, MO, USA) and 5 ng of 17β-E2 in arachis oil were injected s.c. The P4 implants were prepared as described previously (Milligan and Cohen, 1994).

In Study 1, on Days 8 and 9, 5 ng of 17β-E2 in arachis oil was injected s.c. at 09:30 h, while on Day 9 at 11:30 h, 20 μL of arachis oil was injected.

![Figure 1](https://example.com/figure1.png)

**Figure 1** (A) Induction of the mouse menstrual-like model. (B) Assessment of the effect of P4 replacement at 8, 12, 16 or 20 h after the initial P4 withdrawal on endometrial bleeding. (C) Assessment of the effect of P4 replacement at 8, 12, 16 or 20 h on histology and expression of MMPs.
into the lumen of the left uterine horn of each mouse through a dorsal incision to induce decidualization. The right horn was not treated with arachis oil and served as a negative control. The P4 implants were removed 49 h later and mice were sacrificed at 0, 8, 12, 16 and 24 h after P4 withdrawal (n = 8 for each time point) for histomorphology analysis (Fig. 1A). Meanwhile, blood was collected from the orbital sinus of the sacrificed mice to estimate the serum concentrations of P4.

In Study 2, to assess the effect of P4 replacement on endometrial breakdown and bleeding, decidualization was induced as before but P4 implants were replaced at 8, 12, 16 and 20 h or were not replaced after the initial P4 withdrawal (0 h); blank silastic implants were also replaced as controls. Vaginal smear examinations were performed on all mice from 8 to 24 h to monitor endometrial bleedings (Fig. 1B). Mice were sacrificed at 24 h and uterine horns were harvested (Fig. 1C). Blood was also collected from the orbital sinus of mice to estimate the serum concentrations of P4. Mice without successful decidualization in the endometrium on morphologic assessment were excluded from the study. One-half of each horn was fixed in 4% paraformaldehyde for histomorphology analysis, and the other half was frozen immediately in liquid nitrogen before being stored at −70 °C for western blot analysis.

Vaginal smear examination

To monitor endometrial bleeding, vaginal smears from all mice were examined every 4 h from 8 h after P4 withdrawal until the animals were sacrificed. The slides with the vaginal smears were fixed in 95% ethanol for 10 min and then stained with haematoxylin and eosin.

P4 assay

P4 serum levels were determined using radioimmunoassay (Progesterone Radioimmunoassay Kit; BNIBT, Beijing, China). The intra-assay coefficient of variation for P4 was 9%, whereas the inter-assay coefficient was 14%.

Western blot analysis

Before western blot analysis, frozen sections were prepared to assess the status of decidualization and the degree of endometrial breakdown. The cytoplasm of the uterine tissues was extracted using radioimmunoprecipitation assay buffer (Beyotime, Jiangsu, China) with a protease inhibitor cocktail (Merck KgaA, Darmstadt, Germany). The protein concentrations were determined using the Bicinchoninic Acid protein protein assay. In total, 40 μg of proteins was subjected to electrophoresis through 10% SDS–polyacrylamide gels under reducing conditions and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% (w/v) non-fat milk in Tris buffered saline with Tween (TBST) (50 mm Tris–HCl, 150 mm NaCl and 0.05% (v/v) Tween-20, pH 7.6) for 1 h at room temperature and then incubated with primary antibodies (the dilutions and commercial sources of primary antibodies are shown in the Supplementary data, Table SI) overnight at 4 °C. After three washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase-labelled conjugated secondary antibody (1: 10000) for 1 h at room temperature. Proteins in blots were visualized with an enhanced chemiluminescence system (TransGen, Beijing, China) and an X-ray film. The densitometry of the band was analysed with Alpha Imager 2000. β-Actin was used as an internal control to validate the amount of protein loaded onto the gels. Data were presented as a ratio of target proteins versus β-actin.

Statistical analysis

All values were expressed as the mean ± SEM. Statistical analysis of quantitative data was performed by Student’s t-test for significance. The rate of bleeding in mice at each time point was compared between the P4 replacement and control groups using Fisher’s exact test. A P-value of <0.05 was considered significant.

Results

Morphologic changes of the uterus in the mouse menstrual-like model after P4 withdrawal

In stimulated uterine horns, at 0 and 8 h after P4 withdrawal, stromal cells in the functional layer decidualized well, and the whole endometrium was structurally intact (Fig. 2A and B). At 12 h (Fig. 2C), stromal cells were further decidualized compared with at 0 and 8 h. From 0 to 12 h, in a few mice, cell death and haemorrhage occurred in the subepithelium focal stromal zone and red blood cells (RBCs) were present in the uterine cavity (data not shown). At 16 h (Fig. 2D), the decidual tissue was regressing and cells in a larger area of the stroma were dead. Specifically, decidual stromal cells showed nuclear pyknosis or karyorrhexis, cytoplasmic degeneration and a universal lack of discernible cytoplasmic borders, accompanied by bleeding. At 24 h (Fig. 2E), there was a continuous and progressive breakdown, and the most of the decidualized stromal cells were dead and the functional layer was sloughed into the uterine lumen. The above-mentioned changes did not occur in unstimulated uterine horns (Fig. 2F). The number of mice with these typical changes at 0, 8, 12, 16 and 24 h were respectively 7, 8, 6, 7 and 7.

Serum P4 levels were highest at 0 h (128 ± 28.7 ng/mL) (before removing the P4 implant) but were remarkably decreased at 8 h (13.6 ± 4.5 ng/mL, P < 0.01), and remained low thereafter (Fig. 3A).

Effects of P4 replacement on mouse menstrual-like bleeding investigated by vaginal smear monitoring

The presence of RBCs in vaginal smears is an indicator of bleeding in live animals. To evaluate the effect of P4 replacement on endometrial bleeding, vaginal smear examination was performed to monitor the bleeding process in vivo. The degree of endometrial bleeding was classified into three levels by observing the mouse vulva or the vaginal smears either with the naked eye or under a microscope: (i) large amount of bleeding (LAB); large amounts of blood in the vulva were seen with the naked eye (Fig. 4A), (ii) a little bleeding (ALB): RBCs were observed in vaginal smears under a microscope (Fig. 4A) and (iii) no bleeding: no RBCs were seen on vaginal smears under a microscope. LAB mainly occurred from 8 to 12 h and LAB from 16 to 24 h.

In the group with P4 replacement at 8 h, the rate of bleeding (RBC-positive cases against the total inspected in each group) was 12.5% at 8 h after P4 withdrawal; after replacement, the bleedings, including LAB and ALB, disappeared and were completely inhibited compared with the corresponding time points in the control group (P < 0.01 by Fisher’s exact test). In the group with P4 replacement at 12 h, the rate of bleeding was 38.46% and 46.15% at 8 and 12 h, respectively; after replacement, the rate of bleeding was reduced to ≤23.08%, which was evidently inhibited compared with the corresponding time points in the control group (P < 0.01 by Fisher’s exact test). Specifically, the rate of LAB was only 15.38% at 16 and 20 h, and LAB disappeared at 24 h. However, in the group with P4 replacement and control groups using Fisher’s exact test. A P-value of <0.05 was considered significant.
replacement at 16 h, the rates of bleeding from 8 to 16 h increased from 42.86 to 50.00%; after replacement, the rate of bleeding at 20 h was also 50.00%. Bleeding, mainly LAB, was not inhibited compared with the control group (P > 0.05 by Fisher’s exact test). Thus, P4 replacement completely suppressed LAB at 8 h and reduced bleeding at 12 h; however, P4 replacement after 16 and 20 h failed to block bleeding (Fig. 4B and C).

**Effects of P4 replacement on the uterine morphology of the mouse menstrual-like model**

The numbers of mice with successful decidualization in each group are shown in Fig. 4C. In the group with P4 replacement at 8 h (8 of 24 h), the stimulated horns were congested, enlarged and light pink in colour. Histological examination showed that nearly all the stromal cells in the endometrium decidualized and in good state, with no cell death (Fig. 5A1). In the group with P4 replacement at 12 h (12 of 24 h), the morphologic changes were similar to that with P4 replacement at 8 h, and the stimulated horns turned dark in colour (Fig. 5B1). However, in the groups with P4 replacement at 16 and 20 h (16 of 24 and 20 of 24 h, respectively), the stimulated horns were dark red and bleeding had occurred; histology further confirmed that most of the decidualized stromal cells in the stimulated horns were dead and the functional layers underwent breakdown and shedding (Fig. 5C1 and D1). Histological changes also occurred in the control group in which P4 was not restored.

P4 replacement at 8 and 12 h significantly reduced the percentage of endometrial breakdown (the area of endometrial breakdown against the area of decidualization when compared with the control). However, adding back P4 at 16 and 20 h, there was no difference compared with the control (Fig. 5F).

To validate the effectiveness of P4 replacement, the concentration of P4 level in serum was also examined (Fig. 3B). At 24 h, the serum levels of P4 in all mice with P4 replacement were higher than those in mice with no P4 replacement (66.96–102.3 versus 10 ng/ml, P < 0.01).

**Effects of P4 replacement on the expression of MMPs**

MMPs are capable of degrading the extracellular matrix and, thus, are vital factors in endometrial breakdown and shedding. To investigate the reliability of the critical period, the MMP expression patterns in mouse uterus were further explored.

Western blot showed two expression patterns of latent or active MMPs at the protein level. First, the abundance of latent or active MMPs in the P4 replacement group at both 12 and 16 h was significantly reduced compared with that in the control group with no P4 replacement. Specifically, P4 replacement at 12 and 16 h reduced the expression of latent MMP-13 (pMMP-13) (12 of 24 and 16 of 24 h versus 24 h, P < 0.01). Second, the abundance of latent MMP-2 (pMMP-2), latent MMP-3 (pMMP-3), active MMP-9 (MMP-9), latent MMP-10 (pMMP-10) and latent MMP-11 (pMMP-11) in the group with P4 replacement at 12 h was remarkably reduced compared with that in the group with P4 replacement at 16 h and in the control group (12 of 24 h versus 16 of 24 h and 24 h, P < 0.01) (Fig. 6).
Discussion

In this study, a critical period of P4 withdrawal leading to endometrial breakdown and shedding was discovered in a mouse model of menstruation by assessing vaginal smears, morphologic changes and molecular markers.

Vaginal bleeding is an important extrinsic characteristic of endometrial breakdown and shedding in an *in vivo* mouse menstrual-like model. Since P4 replacement after 8 h, completely inhibited endometrial breakdown and P4 replacement at 12 h nearly blocked menstruation, whereas P4 replacement at 16 and 20 h did not block endometrial breakdown (Fig. 4). We can conclude that the critical period of P4 withdrawal for endometrial bleeding in this mouse model was 12–16 h after P4 withdrawal.

Large variations in the initiation of bleeding were observed among individuals (Fig. 4). At 8 and 12 h, the bleeding that was initiated was ALB. ALB also appeared at 0 h, indicating that P4 withdrawal was not strictly necessary to initiate ALB in our study. A previous study also demonstrated that decidual cell death occurred in artificially induced decidua, although the P4 levels were maintained (Gu *et al.*, 1994). Menstruation is triggered by P4 withdrawal (Jabbour *et al.*, 2006); therefore, death of decidual cells accompanied by ALB is not regulated by P4 withdrawal and is not related to the event of menstruation. As in the macaque menstrual model, ALB was excluded, and LAB was our focus in the mouse model in this study. Moreover, although large variations in ALB existed, there was no statistically significant difference in the rate of ALB between the P4 replacement group and the control animals (*P* > 0.05 by Fisher’s exact test). In addition, the rate of bleeding in mice continually increased from 8 to 24 h in the control group, whereas P4 replacement definitely reversed LAB when P4 was replenished at 8 and 12 h, but not at 16 and 20 h. Therefore, a critical period for vaginal bleeding was clearly demonstrated at 12–16 h.

Morphologic assessment further demonstrated that after P4 replacement at 8 and 12 h, stromal cells and the structure between decidual cells were intact, showing blocking of endometrial breakdown and shedding; however, after P4 replacement at 16 and 20 h (Fig. 5), morphologic changes were observed coincident to changes in vaginal smears. Decidual stromal cells were dead, the structure between the stromal cells disappeared, and the functional layer shed from the basal layer accompanied with bleeding; therefore, P4 replacement did not block the endometrial breakdown at these time points. Therefore, the critical period of 12–16 h after P4 withdrawal for endometrial breakdown in the mouse model was confirmed by morphologic changes.

P4 replacement at 12 h clearly inhibited the expression of pMMP-2, pMMP-3, MMP-9, pMMP-10 and pMMP-11; concomitant with the blocking of endometrial breakdown and shedding. P4 replacement at 16 h did not suppress the expression of latent or active MMPs, and neither did it block shedding of the endometrium (Figs 5 and 6). Therefore, by this time, the expression of MMPs was P4-independent and directly associated with the breakdown and shedding of the endometrium. The MMPs investigated in this study are closely associated with menstruation in humans. MMP-2 is present in the proliferative and menstrual phases (Hampton and Salamonsen, 1994); MMP-3 is expressed during menstruation (Hampton and Salamonsen, 1994; Jeziorska *et al.*, 1996); MMP-9, MMP-10 and MMP-11 are present in the late secretory and menstrual phases; and MMP-11 is also found in the proliferative phase (Hampton and Salamonsen, 1994). Generally, MMPs are believed to be the final effectors of endometrial shedding (Jabbour *et al.*, 2006), which is consistent with our results. The inhibition of MMP expression by P4 replacement at 12 h, but not at 16 h, further supports the critical period of 12–16 h in this mouse model.

P4 replacement at 16 h evidently inhibited pMMP-13 expression; however, endometrial breakdown and shedding were not blocked (Figs 5 and 6). This indicates that MMP-13, in contrast to the above-mentioned MMPs, was P4-dependent and not directly associated with endometrial breakdown. MMP-13 was, therefore, functional at an earlier time point than the other MMPs. MMP-13 in mice is the homologue of MMP-1 in humans. MMP-1 is also critical in initiating the degradation of the endometrium and cleaves collagen earlier than other MMPs (Jabbour *et al.*, 2006), which is consistent with our result.
The role of MMP-13 in initiating endometrial breakdown in mice was consistent with the role of MMP-1 in humans, which also appears to support the critical period of 12–16 h in this mouse model. Thus, a critical period of 12–16 h was determined to exist in this mouse menstrual model by assessing vaginal smears, morphologic changes and molecular markers.

P4 withdrawal in an estrogen–P4-primed endometrium occurs with the demise of the corpus luteum as a result of the absence of pregnancy. This triggering event is responsible for the cascade of molecular and cellular interactions that result in menstruation in humans and non-human primates (Nayak et al., 2000; Crichtley et al., 2001; Jabbour et al., 2006). The key role of P4 withdrawal in inducing endometrial breakdown has also been demonstrated in mouse menstrual-like models by physiological and pharmacological P4 withdrawal, and the timing of the induction of endometrial breakdown was determined to be 24–32 h after P4 withdrawal (Finn and Pope, 1984; Brasted...
Further, ours is the first study to report that the critical period of P4 withdrawal induces endometrial breakdown at 24–32 h in a mouse menstrual model, which is also related to P4 regulation. A critical period of P4 withdrawal was also found in a macaque menstrual model. P4 replacement at 36 h could block frank menses in most animals, but replacement at 48 h failed to block menses; thus, the critical period in macaques is 36–48 h after P4 withdrawal. The duration of menstrual sloughing and bleeding in the macaque model is 96 h from P4 withdrawal (Slayden and Brenner, 2006), whereas it is ≏32 h in a mouse menstrual-like model (Xu et al., 2007), which is approximately one-third of the period in macaques. Interestingly, the ratio of the critical period of P4 withdrawal to the time course of menstruation is similar between the mouse and macaque models.

In the macaque menstruation model, P4 replacement at 12–16 h suppressed MMP-1, MMP-2 and MMP-3 expression; however, replacement at 48 h failed to suppress menses—suppressed MMP-1, but not MMP-2, expression. The MMP-1 and MMP-2 expression patterns in the macaque model are concordant with the pMMP-13 and pMMP-2 expression in the mouse model of this study (Slayden and Brenner, 2006). However, P4 replacement at 48 h in the macaque model suppressed MMP-3 expression. This was inconsistent with our study; P4 replacement at 16 h in our mouse menstrual model did not suppress pMMP-3 expression (Slayden and Brenner, 2006). At present, the explanation for this discrepancy in the effect of P4 replacement on MMP-3 expression between these two models is unknown.

The determination of the critical period of P4 withdrawal in a mouse menstrual-like model, as in the primate model, divided the process of P4 withdrawal into P4-dependent and P4-independent stages, which confirms Kelly’s hypothesis and demonstrates the importance of two stages in menstruation. This will aid in the more precise identification of P4-dependent and P4-independent events during endometrial breakdown (Slayden and Brenner, 2006), and simplify studies by limiting the potential factors involved in the process. Differences exist between the mouse and primate models, such as vascular structure and decidualization; moreover, the mouse model requires invasive methods and repeated cycles are troublesome to induce. However, endometrial breakdown occurs in the mouse model (Finn and Pope, 1984; Brasted et al., 2003; Xu et al., 2007; Rudolph et al., 2012). As a mammalian animal model, the mouse model is superior to the primate model with respect to the availability of animals, number of ethical considerations, available methods of manipulation in vivo and defined genetics. Therefore, determining the critical period of P4 withdrawal in the mouse model could clarify...
P4-regulated signalling pathways, as well as those not regulated by P4, in the process of endometrial breakdown using high-throughput techniques. This will be the focus of further investigation in our laboratory.

The existence of a critical period for P4 withdrawal divides the process of P4 withdrawal into two stages (P4-dependent and P4-independent) in this mouse menstrual-like model. Understanding P4-regulated events and those not regulated by P4 will provide new insight into the mechanisms underlying menstruation and identify new targets for the clinical intervention of menstrual disorders.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles
X.X., B.H. and J.W. conceived and designed the experiments; Q.W., X.X. and B.H., Y.L., X.C. performed the experiments and X.X., B.H. and J.W. analysed the data.

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Conflict of interest
None declared.

References


Finn CA, Pope M. Vascular and cellular changes in the decidualized endometrium of the ovariectomized mouse following cessation of

Figure 6 Analysis of the effect of P4 replacement at 12 and 16 h on the amount of pMMP-2, pMMP-3, MMP-9, pMMP-10, pMMP-11 and pMMP-13 present after 24 h measured by western blot. ‘pMMP’ refers to the pro MMP, which corresponds to latent MMP; ‘MMP’ refers to the active forms. Bar graphs show the results of densitometric analysis. The expression of protein in different P4 replacement groups was compared by Student’s t-test. **P < 0.01, 12 of 24 h versus 16 of 24 h and 24 h. ## P < 0.01, 12 of 24 and 16 of 24 h versus 24 h.


