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Effect of mifepristone on uterine receptivity in guinea pigs[1]

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KEY WORDS mifepristone; endometrium; guinea pigs; cytokines

ABSTRACT

AIM: To determine the effect of a low-dose mifepristone regimen on endometrial receptivity in guinea pigs. **METHODS**: Histologic evaluation for endometrial staging and immunoreactivity of cytokines [leukemia inhibitory factor (LIF) and interleukin-6 (IL-6)] were preformed on fixed endometrial tissue, and relative abundance of endometrial estrogen (E), and progesterone (P) receptor mRNA were evaluated with semiqantitative reverse transcriptase polymerase chain reaction (RT-PCR) in which cyclophilin mRNA, a housekeeping gene product, was co-amplified as the reference standard. **RESULTS**: Mifepristone at 0.03 $mg \cdot kg^{-1} \cdot d^{-1}$ induced a delay in endometrial maturation. The staining intensity of LIF and IL-6 decreased in treated guinea pigs. E and P receptor mRNA increased significantly and uterine stage delayed in treated guinea pigs. **CONCLUSION**: Mifepristone at 0.03 $mg \cdot kg^{-1} \cdot d^{-1}$ delayed development of endometrium. This study provides further evidence that endometrial maturation can be altered without affecting ovarian ovulatory function.

INTRODUCTION

Uterine receptivity is dependent on the endometrial decidua tissue formation and maturation. The induction and extent of decidual tissue formation are well recognized to be dependent on both the proper ovarian steroid hormone priming of the endometrium and the type of natural (ie, blastocyst) or experimental (eg, chemical or mechanical) stimulus applied to the uterine stroma[1,2]. Endometrial development is characterized by a series of structural and morphologic changes of the endometrium. Estrogen (E) and progesterone (P) induce these proliferative and secretory changes[3,4]. However, autocrine and paracrine regulators, collectively termed cytokines, appear to be involved in controlling these endometrial functions. Many cytokines, including leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) are expressed in the human endometrium in a menstrual cycle-dependent manner.

Mifepristone is a synthetic steroid that blocks the biologic effects of P at the receptor level. Recent reports suggest that chromic administration of low doses of mifepristone can alter endometrial development and function while maintaining ovarian function and menstrual cyclicity[1]. Grabam *et al*[5] reported that a single dose of mifepristone given on LH - 2 (where LH + 0 was the LH peak, ie, the day of ovulation) prevented the appearance of a luteal-specific secretory glycan. In our previous study, differential effect on ovary function was observed in the dose range of 0.01 - 8.0 mg/kg body weight. The dose range of 0.01 - 0.05 mg/kg did not disrupt the ovarian function. So the dose at 0.03 mg/kg was used in this study.

The guinea pig is a good model to study human implantation. The estrous cycle of the guinea pig is similar to the human menstral cycle in that it is relatively long and includes the development of a functional corpus luteum which secretes P, so that the uterus is dominated by hormonal conditions similar to early pregnancy. In the normal cycle in guinea pig and human, the hormonal condition of pregnancy prevails without the complication in the presence of an embryo. The mode of implantation in guinea pig is also similar to that in human.

The objective of this study was to determine the effects of a low-dose regimen of mifepristone (0.03 $mg \cdot kg^{-1} \cdot d^{-1}$) at the endometrial level in normally cycling guinea pigs. To complete this objective, we used histochemical techniques for endometrial staging and immunocytochemistry to detect region-specific alterations in the LIF and IL-6 in the endometrium. We used

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semiquantitative reverse transcriptase PCR in which cyclophilin mRNA, a housekeeping gene product, was co-amplified as the reference standard to compare the relative abundance of E receptor (ER) and P receptor (PR) mRNA in the control and mifepristone-treated endometrium.

MATERIALS AND METHODS

Treatment of animals Adult female virgin guinea pigs (500 – 600 g, purchased from Cao-hua Company, Grade Ⅱ, Certificate № 135, Shanghai, China) were maintained under controlled environmental conditions with an established photoperiod of 12 h light/day. Food and water were available *ad libitum*. Daily vaginal examination was used to monitor reproductive cycles, with vaginal patency in association with cornified cells in a lavage used to denote d 1 of the estrous cycle. Twenty guinea pigs demonstrating cycle regularity were randomly placed in treatment and control groups. Ten guinea pigs were treated with mifepristone (0.03 $mg \cdot kg^{-1} \cdot d^{-1}$ in sesame oil) beginning on d 2 of estrous cycle for 12 d and ten guinea pigs treated with the vehicle (sesame oil) for the same time period. At the end of treatment, the guinea pigs were euthanized and their uteri were harvested. One uterous was fixed with 10 % formalin for histologic and immunocytochemical studies. Another uterus was snap-frozen in liquid nitrogen and stored at – 80 ℃ until mRNA isolation for reverse transcriptase PCR.

Endometrial assessment Blocks of endometrium that had been fixed in 10 % buffered formalin were embedded in paraffin. Morphological examination of endometrium under microscope after histological and hematoxylin and eosin staining. Morphological criterias included endometrial thickness, grading of glandular secretion, subnuclear vacuoles, stromal edema, and coiled spiral arteries.

Immunohistochemistry The 4 – 6 μm thick sections were mounted on 3-amino-propyl-tri-ethoxy-silane (APES)-coated glass slides. For antigen retrieval the sections were heated four times (each 5 min) in citric acid butter using amicroware oven (600 W). After they were allowed to cool down for about 30 min, the sections were placed in 0.3 % H_2O_2/methanol for 30 min to block endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA), the sections were blocked for non-specific binding of the secondary antibody by an incubation with normal rabbit serum (1:20) in the case of LIF and IL-6. Sections were incubated with the monoclonal anti-human LIF antibody or mouse IL-6 antibody in a dilution of 1:4 in PBS/0.1 % BSA for 90 min at 37 ℃. After an additional wash with PBS/0.1 % BSA the sections were incubated with the biotinylated secondary antibody (goat anti-mouse). The antibody binding was detected by the biotin-streptavidin peroxidase method using a streptavidin-peroxidase solution for 20 min at 25 ℃ twice to intensify the immunohistochemical signal. Subsequently antibody-binding was detected by immersing the sections for 5 – 10 min in a solution of the chromogen amino-ethyl-carbazole. The slides were washed with PBS and deionized H_2O and embedded with glycerol gelatine without previous counterstaining. Negative control was performed by using normal rabbit serum at a 1:20 dilution instead of the primary antibody. Each staining was repeated twice and was scored blindly by two observers. Each sample was scored using the noncounterstained sections from 0 (negative) to 3 (maximal staining intensity).

Reverse transcriptase PCR polyA RNA was obtained by oligo (dT) chromatography with use of a commercially available kit (Micro-Fast Track, Invitrogen, San Diego, CA) follow the manufacture's specifications and as modified by Heikinheimo *et al*[6]. Polyacrylamide gel (7 %) electrophoresis was used to isolate each PCR product, which was then visualized with ethidium bromide staining. The relative abundance of co-amplified PCR products was measured by scintillation spectroscopy of excised PCR-amplified fragments. Data were expressed as the Bq ratios of ER or PR to cyclophilin mRNA. Counts (Bq) of the control sample, processed without reverse transcriptase, were used as background.

Statistical analysis All results are presented as $\bar{x} \pm s$. Data were analyzed by linear regression analysis, Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Histologic evaluation Histologic assessment of endometrial tissue showed a delay of maturation in all of the mifepristone-treated guinea pigs (Fig 1B). In contrast, the endometrium of most control guinea pigs (9/10) was determined to be in the secretory phase (Fig 1A). A delay in the endometrial characteristics, endometrial thickness, glandular secretion, subnuclear

vacuoles, stromal edema, and presence of coiled spiral arteries was consistent with the delayed endometrial stage observed in the mifepristone treated guinea pigs compared with the control group.

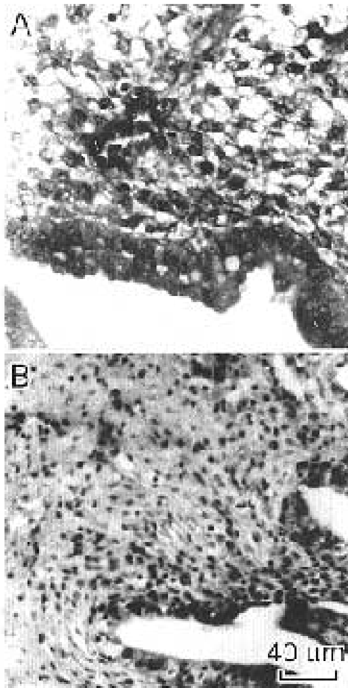

Fig 1. Histologic assessment of endometrial tissue from the control (A) and mifepristone-treated (B) guinea pigs, the mifepristone- treated endometrium showed a delay of endometrial maturation. The endometrium of control guinea pigs was determined to be in secretory phase and showed glandular secretion, subnuclear vacuoles, and stromal edema. ×400.

Immunocytochemistry Positive immunoreactivity for LIF (Fig 2A – C) and IL-6 (Fig 2D – F) was observed in endometrium tissues of all control and treated guinea pigs. A low (score = 1) to moderated (score = 2) level of immunoreactivity was observed throughout the endometrial stroma. Stronger (score = 3) immunostaining was observed in the glandular and luminal epithelium compared with the stromal region. Both LIF and IL-6 were localized intracellularly to the cytoplasm and nucleus, immunoreactivity was also detected in the lumen of the glands. For LIF and IL-6, with the endometrial characteristics, the staining intensity was consistent with the endometrial stage.

The endometrium from the nine guinea pigs in control group that was staged as late secretory exhibited the strongest intensity of staining in the glandular and luminal epithelium (Fig 2A and D) (score = 3) > immunoreactivity of LIF and IL-6 in the endometrium of the mifepristone-treated guinea pigs was moderated (score = 2), not reaching the level of intensity observed in the control endometrial tissues (Fig 2B and E respectively). No staining was observed when LIF and IL-6 antisera had been pre-absorbed with recombinant LIF (Fig 2C) and IL-6 (Fig 2F), respectively.

Abundance of ER and PR mRNA Fig 3 shows a representative ethidium bromide-stained polyacrylamide gel of reverse transcribed mRNA co-amplified for ER and PR with cyclophilin. Within each set of complification reactions, there was no difference in cyclophilin mRNA levels as determined by the signal intensity of excited bands from the control and treated endometrial tissues. Therefore, the results of the co-amplification reactions to examine the relative abundance of the ER and PR transcriptions are expressed as the Bq ratios of ER to cyclophilin of PR to cyclophilin. Treatment of guinea pigs with low-dose mifepristone resulted in a statistically significant increase in both the ER to cyclophilin and PR to cyclophilin ratio ($P < 0.05$) (Fig 4). No DNA could be detected in the negative controls.

DISCUSSION

The regulation of implantation is not clearly understood, however, P is essential for a receptive endometrium. The development of antiprogestin, which blocks the action of P in its target organs, has offered a way to study P-dependent processes involved in successful implantation, inducing a receptive endometrium[7].

The effect of mifepristone is dependent on both the dose and the timing of treatment. Initial studies found that mifepristone administration in large doses ($8\ mg \cdot kg^{-1} \cdot d^{-1}$) or in the mid ($4\ mg \cdot kg^{-1} \cdot d^{-1}$) to late follicular phase delayed the LH surge, postponed ovulation, and induced premature menstruation in the mid-luteal phase, whereas late luteal-phase administration prevented pregnancy[8,9]. Recent reports have indicated that chronic treatment of rhesus monkeys and women with low doses of an antiprogestin interfered with uterine receptivity with no alteration in menstrual cyclicity[10,11]. This differential effect on the ovary and the endometrium was observed in the dose range of 0.1 – 1.0 mg/kg body weight[12,13].

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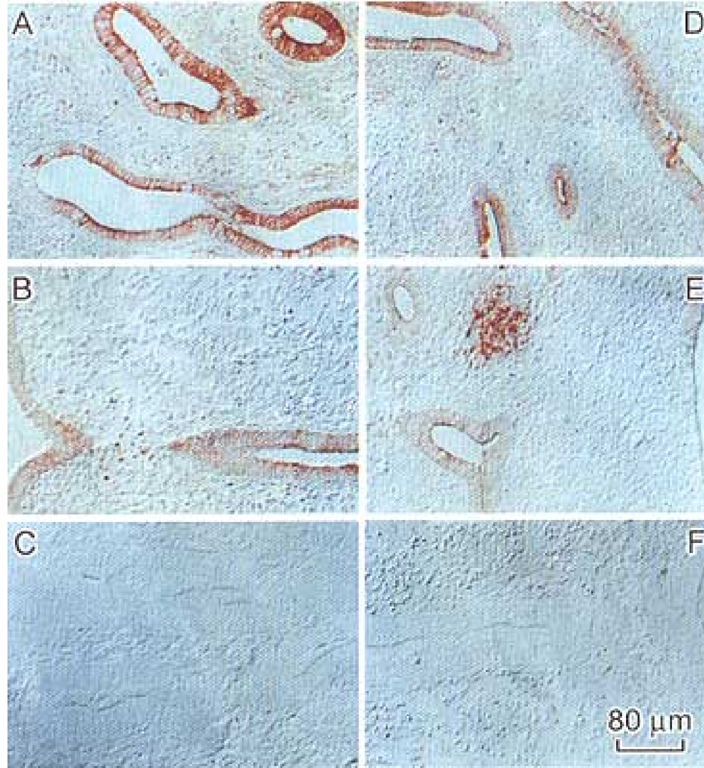

Fig 2. Immunohistochemical localization of LIF (A, B, C) and IL-6 (D, E, F) in control (A, D) and low-dose mifepristone treated (B, E) guinea pig endometrium. Pre-absorption of the LIF and IL-6 antisera with recombinant LIF (C) and IL-6 (F), respectively, also served as the negative control. A strong staining (score = 3) detected in control endometrium. Mifepristone reduced the staining of LIF and IL-6 to low levels (score = 1 - 2). ×200.

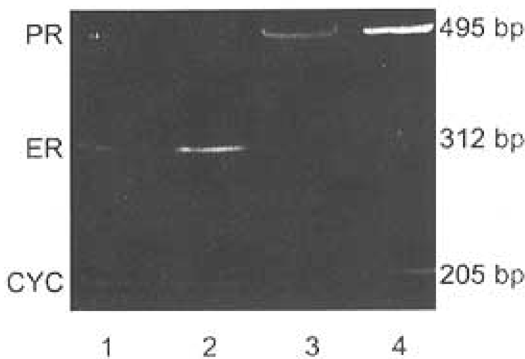

Fig 3. Representative ethidium-bromide-stained polyacrylamide gel of reverse transcribed ER and PR mRNA co-amplified by PCR with cyclophilin (CYC) mRNA from endometrial tissue of control and mifepristone-treated guinea pigs. The expected products from the ER, PR, and CYC migrate at 205, 312, and 495 bp, respectively. 1, control; 2, +mifepristone; 3, control; 4, +mifepristone.

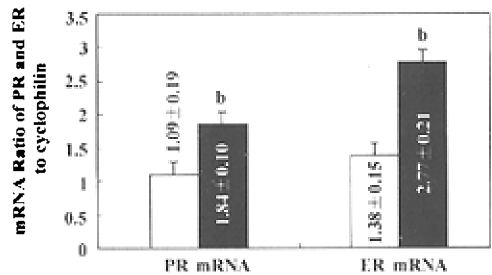

Fig 4. Effect of low-dose mifepristone (■) compared with vehicle control (□) on endometrial ER and PR mRNA abundance. Data are presented as $\bar{x} \pm s$ of the ratio of relative abundance of each mRNA with cyclophilin (CYC). $n = 8$. ${}^{b}P < 0.05$ *vs* vehicle control group.

In this study, mifepristone at 0.03 $mg \cdot kg^{-1} \cdot d^{-1}$ delayed the endometrial maturation. Previous studies have indicated that adminstration of mifepristone at dose of 1 $mg \cdot kg^{-1} \cdot d^{-1}$ during d 2 - 22 resulted in

suppression of the endometrium with few mitoses, dense stroma, and simple glands. In the present study, a similar dose of mifepristone was administrated to guinea pigs, with similar results. Although the effect of mifepristone at this dose showed no effects on presumed ovulation, it affected uterine receptivity.

Both LIF and IL-6 are expressed in the human endometrium in a menstrual cycle-dependent manner, with maximal expression observed between d 19 and 25, the time considered to be the window of implantation[14-17]. It has been suggested that LIF acts on human trophoblasts to shift their differentation toward the anchoring phenotype by increasing the synthesis of fibronectin and decreasing the production of β-hCG[18], IL-6 may be involved in a similar manner because the two cytokines share a common signal transducer[19,20].

In the present study, immunoreactivity of these two cytokines was compared between the control and treated endometria. Mifepristone decreased the expression of both in guinea pig endometrium. The mRNA levels for both receptors were significantly higher in the treatment than those in the control guinea pigs. Mifepristone appears to be the most potent known antiprogesterone and antiglucocorticoid observed both *in vitro* and *in vivo*. In contrast, it also provokes permanent estrus after chronic administration to intact rats at doses of 10 mg/kg and above. Under the same experimental conditions, the compound does not display antiovulatory activity but seem to stimulate the pituitary ovarian axis. In various species, mifepristone also exhibited slight uterotrophic activity (30 - 300 mg/kg). This always occurred, even in ovariectomized and adrenalectomized rats. These results suggested that this uterine weight increase was mediated by a transient interaction between mifepristone and the uterine estrogen receptor. It may be the cause of that mifepristone increased the mRNA levels for both receptors. As with the alteration observed for the endometrial cytokine levels, this increase in ER and PR mRNA abundance indicates a delay in endometrial maturity. However, whether this increase in mRNA levels after low dose of mifepristone treatment is a direct result of a delay in endometrium or reflects an independent action remains to be determined. However, this study provides further evidence on a molecular and cellular level that endometrial maturation can be altered without affecting ovulatory function.

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米非司酮对豚鼠子宫内膜接受性的影响[1]

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关键词 米非司酮; 子宫内膜; 豚鼠; 细胞因子

目的: 从细胞及分子水平分析低剂量米非司酮在子宫内膜对胚泡接受性中的作用. **方法**: 以组织学方法观察内膜形态变化; 免疫组织学方法测定 LIF 及 IL-6 在子宫内膜的基因表达以及用半定量反转录—聚合酶链反应(RT-PCR)测定内膜雌、孕激素受体 mRNA 水平等方法来评估子宫内膜的成熟度. **结果**: 米非司酮在剂量为 0.03 $mg \cdot kg^{-1} \cdot d^{-1}$时, 可明显延缓子宫内膜的成熟、抑制间质细胞蜕膜化, 降低 LIF 和 IL-6 在内膜的基因表达和增高内膜雌、孕激素受体 mRNA 水平. **结论**: 小剂量米非司酮可直接作用于子宫内膜, 影响内膜的成熟而抑制内膜对胚泡的接受性.

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