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Mifepristone regulates expression of apoptosis related genes *Fas* and *FasL* in mouse endometrium[1]

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KEY WORDS mifepristone; apoptosis; *in situ* hybridization; immunohistochemistry

ABSTRACT

AIM: To investigate the anti-implantation mechanism of mifepriston. **METHODS**: *In situ* hybridization and immunohistochemistry were applied to determine mRNA and protein. **RESULTS**: After mifepriston injection, the number of implantation sites were obviously reduced, mifepriston could inhibit the embryo implantation in mouse. The expression of apoptosis related genes, *Fas* and *FasL*, in mouse endometrium was also decreased after mifepriston treatment. **CONCLUSION**: The expression of apoptosis related genes *Fas* and *FasL* is regulated by mifepriston and the inhibitory effect of mifepriston on the embryo implantation may be mediated by action on the *Fas/FasL* system.

INTRODUCTION

Mifepriston is an 11β-dimethyl-amino-phenyl derivative of norethindrone with a high affinity to progesterone and glucocorticoid receptors, but it does not result in the transcription of the progesterone-dependent gene. Mifepriston has been widely adopted in clinics as an abortive drug to terminate early pregnancy. Much evidence showed that administration of mifepriston after ovulation could prevent the embryo implantation in primates[1], the underlying mechanism, however remains unknown. The term apoptosis describes the model of cell death that is responsible for the deletion of cells in the normal tissues[2]. The process is distinct from necrosis, it is a normal physiologic phenomenon and displays characteristic structural changes in the nucleus and cytoplasm, such as the fragmentation of DNA. The most important point of the apoptotic process is that it is regulated by some related genes. Some genes, such as *bcl-2*, inhibited apoptosis[3], while some others, such as *Fas* and *FasL*, promoted this process. *Fas* is a type Ⅰ membrane protein that belongs to the tumor necrosis and nerve growth factor receptor family[4,5]. *FasL* is a type Ⅱ membrane protein also related to the tumor necrosis factor family[6]. *FasL* can either be membrane bound (M_r = 42 - 48) or be cleaved by metalloproteinases to release the extracellular portion as soluble *FasL* (M_r = 26). The major function of *Fas/FasL* interaction and *Fas* activation is the induction of cell apoptosis[4]. There are many reports related to the apoptosis in the endometrium before implantation, but the physiologic significance of this phenomenon and the factors involved in this process, however, have not yet been identified. von Rango *et al*[7] suggested that the apoptosis in the endometrium was a marker of the endometrium receptivity, and this phenomenon might be regulated by progesterone[8]. Therefore, it is possible that the anti-implantation effect of RU486 may be mediated by *Fas/FasL* system. To test this hypothesis, the mice were injected with various doses of mifepriston after ovulation, the number of implantation sites was analysed and the expression of *Fas/FasL* and the fragmentation of DNA in the tissue were examined.

MATERIALS AND METHODS

Animal and regent Adult female Kunming mice were purchased from Experimental Animal Institute of Chinese Academy of Medical Sciences (Grade Ⅱ, Certificate № 01 - 3001); The restriction enzyme and TdT were purchased from Promega; NBT, BCIP, anti-DIG antibody, blocking reagent, and dig-ddUTP were purchased from Boehringer. RU486 were purchased from the Third Pharmaceutical Factory of Beijing. *Fas* and *FasL* polyclonal antibody, biotin-conjugated secondary

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antibody, normal goat serum, ABC kit, and DAB kit were purchased from Santa Cruz.

Animal treatment Mice were divided into three groups and the presence of vaginal plug after mating was designated as d 1 of pregnancy. In the afternoon of d 1 and d 2, two doses of mifepriston, 4 and 12 mg/kg (body weight) were injected intra-peritoneal (ip) in two groups respectively. The control group was injected with the vehicle (benzyl/olive oil 1:4) only. On d 4 afternoon, the mice were killed, and the uteri and the ovaries were fixed in 4 % formaldehyde solution and cryopreserved at −70 ℃ for immunohistochemistry. Some animals were killed on d 6, the implantation sites were counted and the effect of two doses of mifepriston were checked on implantation.

***In situ* hybridization** Probe labeling was based on the method of Liu *et al*[9], *in situ* hybridization was carried out based on the method of Schaeren-Wiemers and Gerfin-Moster[10].

Immunohistochemistry The dried sections were fixed in 4 % paraformaldehyde in PBS (5 min), washed with PBS and endogenous peroxidase activity was quenched by incubating sections in 3 % H_2O_2 in methanol (10 min). The sections were washed with PBS, and pre-incubated with normal goat serum blocking solution (1:20 diluted with PBS) for 20 min. Sections were incubated with *Fas* and *FasL* polyclonal antibody (1:100 in blocking solution) for 1 h. Then the sections were washed with PBS (3 × 5 min) and the slides were incubated with biotin-conjugated secondary antibody (1:200 in blocking solution) for 45 min. Sections were washed with PBS (3 × 5 min), then the slides were incubated for 45 min with a mixture of reagent A (avidin) and B (biotin) prepared 30 min in advance with PBS (1:100 for each reagent). Sections were washed thoroughly with PBS and incubated with DAB substrate solution for 2 − 7 min, the nuclei of the tissue were counter-stained using haematoxylin.

***In situ* 3′-end-labeling of fragment DNA of apoptotic cells in the mouse endometrium** *In situ* analysis was performed as described previously[11,12]. Deparaffinized and hydrated samples were then treated with proteinase K. Samples were then subjected to 3′-end-labeling of the DNA with digoxigenin-dideoxy-UTP. The standard substrates BCIP and NBT were used for staining.

Western blot To perform Western blot analysis for *Fas*, intact mouse uteri were homogenized in buffer (Tris-HCl 10 mmol/L, NaCl 100 mmol/L, edetic acid 1 mmol/L, PMSF 100 mg/L). The homogenate was clarified by centrifugation, and total protein content of the supernate was determined by a Coomassie protein using albumin as the standard. For *Fas* Western blot analysis, 50 μg of total cellular protein was fractionated in a denaturing 10 % SDS-polyacrylamide gel by electrophoresis. The fractionated samples were then transferred to nitrocellulose membrane, and nonspecific binding was blocked for 2 h in 5 % nonfat dry milk in PBS. The membranes were incubated with anti-*Fas* antibody for 1 h (1:200). The membranes were washed in PBS, then incubated with AP-conjugated secondary antibody for 1 h, after washing with PBS, the membranes were incubated with CDP-star for 10 min. Then the membranes were exposured to X-ray film.

Statistical analysis The data were obtained from at least three independent experiments. The values were expressed as $\bar{x} \pm s$. Statistical analysis was performed using unpaired t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of mifepriston on embryo implantation As shown in Tab 1, mifepriston inhibited the embryo implantation in mice, as compared with the control group.

Tab 1. The effect of mifepriston on the number of implanted embryos in mice. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Dose/ mg·kg^{-1}	Number of animals	Number of implantation sites	Implanted embryos
Control	6	86	14.3 ± 1.0
Mifepriston 4	7	51	7.3 ± 2.2^c
Mifepriston 12	6	3	0.5 ± 1.0^c

Expression of *Fas* and *FasL* mRNA in the endometrium The *Fas* mRNA were highly expressed in both glandular epithelial and stromal cells as shown in the control group (Fig 1a). On treatment with low dose mifepriston (4 mg/kg), the expression of *Fas* mRNA decreased considerably (Fig 1b), and the *Fas* mRNA was almost not detected in the group treated with high dose of mifepriston (12 mg/kg) (Fig 1c). The expression of *FasL* mRNA in the two groups was similar to that of the *Fas* mRNA (Fig 2).

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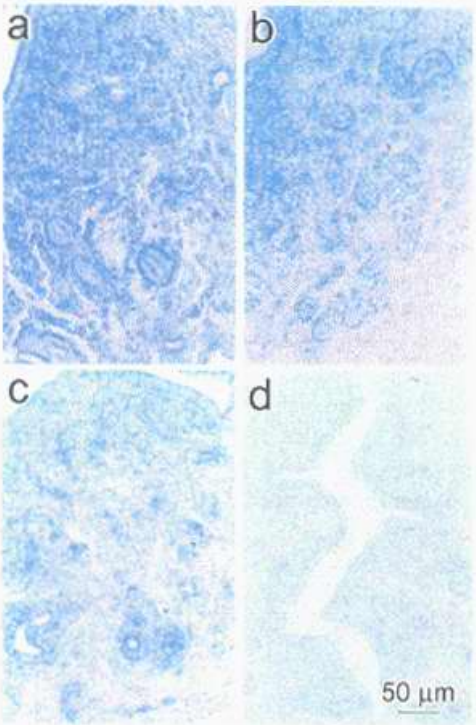

Fig 1. Localization of *Fas* mRNA in the endometrium of mice on d 4 after ovalation. (a) Control group; (b) Mifepriston 4 mg/kg; (c) Mifepriston 12 mg/kg; (d) Negative control.

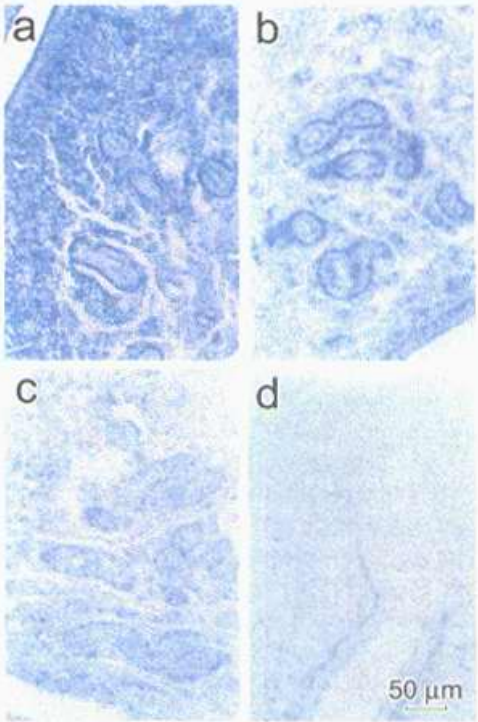

Fig 2. Localization of *FasL* mRNA in the endometrium of mice on d 4 after ovalation. (a) Control group; (b) Mifepriston 4 mg/kg; (c) Mifepriston 12 mg/kg; (d) Negative control.

Distribution of Fas and FasL antigen in the endometrium The distribution pattern of Fas and FasL antigen were similar to the expression of *Fas* and *FasL* mRNA. After mifepriston treatment, the expression of both proteins decreased remarkably compared with the control (Fig 3b and Fig 4b). There was no positive signal of Fas and FasL antigen in glandular epithelium as indicated by the arrows in Fig 3 and Fig 4.

Western blot analysis of the expression of Fas antigen The result of Western blot is consistent with that of *in situ* hybridization and immunohistochemistry. After mifepriston injection, the expression of Fas antigen decreased obviously (Fig 5).

***In situ* analysis of DNA fragmentation in the endometrium** In order to histologically examine the apoptotic cells in the endometrium, the experiment of *in situ* 3′-end-labeling of DNA fragment of apoptotic cells was conducted. The results indicated that in the endometrium of control group there were a large number of apoptotic cells, mainly localized in the glandular epithelium (Fig 6a). On treatment with mifepriston the number of apoptotic cells were decreased (Fig 6b and Fig 6c). This result is consitent with the *in situ* hybridization and immunohistochemistry results.

DISCUSSION

As a potential contraceqtive drug, mifepriston attracts the attention of many researchers, focusing mainly on its anti-pregnancy effect. Studies on the anti-implantation action og mifepriston are very limited. In this study, we first systematically investigated the anti-implantation effect of mifepriston using mouse as a model. The results showed that mifepriston could inhibit embryo implantation. Low dose (4 mg/kg) was observed to just partly prevent the embryo implantation, but on treatment with high dose (12 mg/kg), almost all the embryos could not be implanted into the endometrium. On the other hand, *in situ* hybridization and immunohistochemistry results indicated that the expression of apoptosis related genes *Fas* and *FasL* also was decreased after mifepriston treatment, thus it was reasonable to suggest

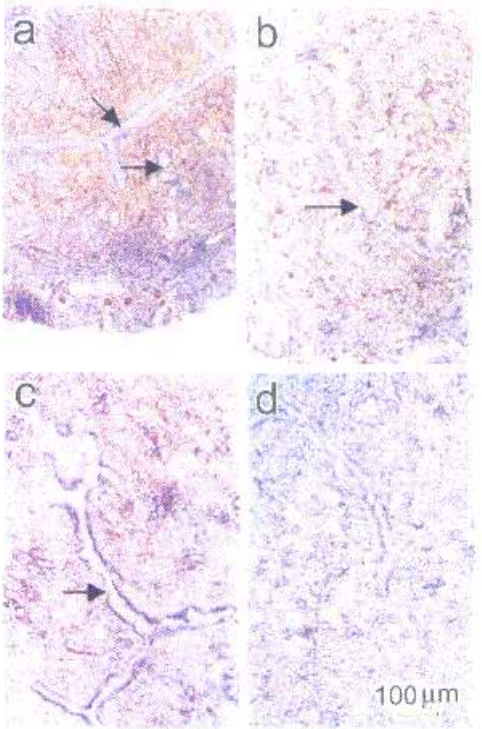

Fig 3. Immunolocalization of *Fas* protein in the endometrium of mice on d 4 after ovulation. (a) Control group; (b) Mifepriston 4 mg/kg; (c) Mifepriston 12 mg/kg; (d) Negative control.

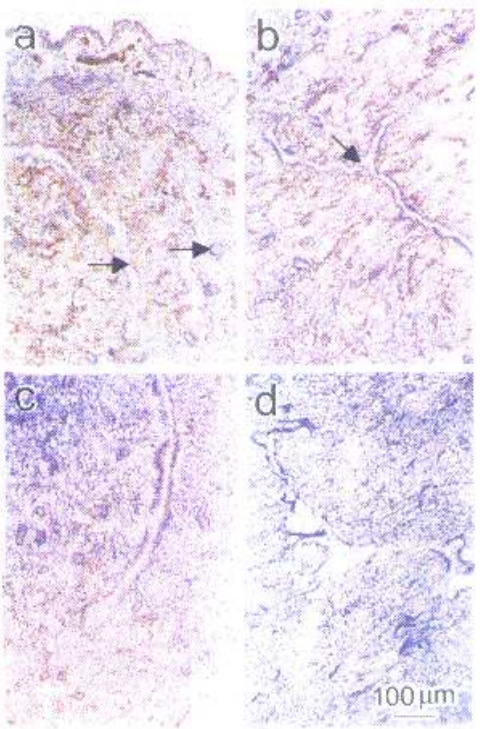

Fig 4. Immunolocalization of *FasL* protein in the endometrium of mice on d 4 after ovulation. (a) Control group; (b) Mifepriston 4 mg/kg; (c) Mifepriston 12 mg/kg; (d) Negative control.

that there was relationship between the endometrium receptivity and the expression of *Fas* and *FasL*. Thus the anti-implantation effect of mifepriston might be mediated by *Fas/FasL* system.

It is generally believed that the implantation window in mouse opens up on d 4 to d 5 after ovulation and maintains for about 24 h[31]. In this study, we defined the optimal uterine receptivity period. The results of *in situ* hybridization and immunohistochemistry showed that *Fas* and *FasL* mRNA and proteins were highly expressed in the endometrium of normal pregnant group, and the result of TUNEL also suggested that a large number of cells underwent apoptosis in the same tissue, this result was consistent with a previous report[34]. Apoptosis plays an important role in the tissue differentiation and remodeling[15,16], and may be regarded as a marker of uterine receptivity. It has been suggested that apoptosis may provide a mechanism explaining maternal immune tolerance to the fetus during the process of implantation[17-19]. *Our experiments demonstrated that the expression of Fas* and *FasL* as well as the number of apoptotic cells sharply

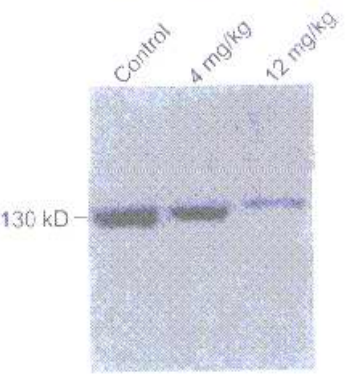

Fig 5. Western blot analysis of *Fas* protein.

decreased on treatment with mifepristen, and at the same time the number of implantation sites greatly decreased also. These results suggested that RU486 was capable of changing the uterus receptivity and might exert anti-implantation effect through *Fas/FasL* system. However, the exact mechanism is poorly understood.

In every estrous cycle, the oestrogen and proges-

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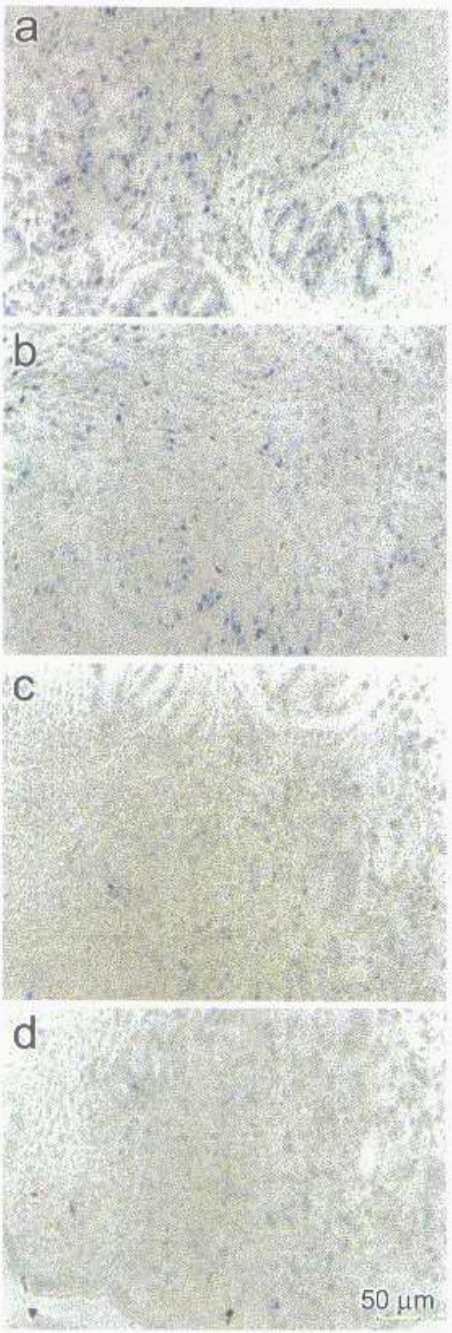

Fig 6. ***In situ*** **3′-end-labelling of DNA in the section of mouse endometrium on d 4 after ovulation. Dig-ddUTP labeling was represented by the blue coloration and such staining was the result of the apoptotic cleavage of DNA. (a) Control group; (b) Mifepriston 4 mg/kg; (c) Mifepriston 12 mg/kg; (d) Negative control.**

terone are secreted cyclically, and these two hormones synergistically regulate the proliferation and differentiation of the endometrium. Estrogen mainly promotes the cell proliferation while progesterone simulates the estrogen-primed cell differentiation. Mifepriston binds to the progesterone receptor to block the progesterone function, therefore the differentiation is retarded and the uterus still maintains a proliferating condition. Thus it is feasible that the expression of the apoptosis-related genes *Fas* and *FasL* is inhibited resulting in decreased uterine receptivity.

In conclusion, the apoptosis occuring in the endometrium during uterine receptivity period is an important condition for embryo implantation. The anti-implantation effect of mifepriston may be mediated through the down-regulation of the *Fas* and *FasL* expression.

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米非司酮调控凋亡相关基因 *Fas* 和 *FasL* 在小鼠子宫内膜中的表达[1]

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关键词 米非司酮；细胞凋亡；原位杂交；免疫组织化学

目的：研究米非司酮抗着床机理. **方法**：用原位杂交法检测 mRNA 的表达，用免疫组化法定位蛋白质. **结果**：注射米非司酮后，胚胎的着床数明显下降，另外，注射米非司酮后与凋亡相关的基因 *Fas* 和 *FasL* 的表达也受到抑制. **结论**：米非司酮调控凋亡相关基因 *Fas* 和 *FasL* 的表达，且其抗胚胎着床作用可能是由 *Fas*/*FasL* 介导的.

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