

## Mifepristone regulates expression of apoptosis related genes *Fas* and *FasL* in mouse endometrium<sup>1</sup>

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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 11 $\beta$ -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<sup>[1]</sup>, 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<sup>[2]</sup>. The process is distinct from necrosis, it is a normal physiologic phenomenon and displays char-

acteristic 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<sup>[3]</sup>, 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<sup>[4,5]</sup>. *FasL* is a type II membrane protein also related to the tumor necrosis factor family<sup>[6]</sup>. *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<sup>[4]</sup>. 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*<sup>[7]</sup> suggested that the apoptosis in the endometrium was a marker of the endometrium receptivity, and this phenomenon might be regulated by progesterone<sup>[8]</sup>. 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 reagent** Adult female Kunming mice were purchased from Experimental Animal Institute of Chinese Academy of Medical Sciences (Grade II, Certificate No 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  $-70^{\circ}\text{C}$  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*<sup>(9)</sup>, *in situ* hybridization was carried out based on the method of Schaeren-Wiemers and Gerfin-Moster<sup>(10)</sup>.

**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 %  $\text{H}_2\text{O}_2$  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  $\times$  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  $\times$  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<sup>(11,12)</sup>. 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  $\mu\text{g}$  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 exposed 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.  $\bar{x} \pm s$ . \* $P < 0.01$  vs control.

Dose/ $\text{mg} \cdot \text{kg}^{-1}$	Number of animals	Number of implantation sites	Implanted embryos
Control	6	86	$14.3 \pm 1.0$
Mifepriston 4	7	51	$7.3 \pm 2.2^*$
Mifepriston 12	6	3	$0.5 \pm 1.0^*$

### 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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evolutionary perspective. Hum Reprod 1996; 11: 283-6.

### 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<sup>1</sup>

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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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